# RENAL EXCRETION OF POTASSIUM IN NORMAL AND SODIUM DEPLETED DOGS [1]

By HELEN M. ANDERSON and JOHN H. LARAGH with the technical assistance of CLARA W. HALL, SALLY MOORE, and JOSEPH HARTOG

*(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and The Presbyterian Hospital in the City of New York, N. Y.)*

(Submitted for publication July 26, 1957; accepted October 31, 1957)

There is considerable evidence indicating that the renal loss of potassium is reduced in both man and animals following the establishment of a sodium retaining state. Seldin, Welt, and Cort (1) and Relman and Schwartz (2) have demonstrated that a low sodium diet limits the excretion of potassium in patients and in rats receiving either desoxycorticosterone (DCA) or hydrocortisone. In addition, Milne, Hughes Jones, and Evans (3), studying normal individuals on a low potassium diet, found that the renal conservation of this ion was more efficient when the sodium intake was also reduced. Dogs forming ascites after thoracic caval constriction have been shown to retain nearly all ingested sodium, and frequently to exhibit hyperkalemia. When these animals were given DCA, the expected kaliuresis did not occur (4). It has been postulated that the failure of kaliuresis in these and other similar circumstances is secondary to a low sodium content of distal tubular urine. Such a reduction in sodium content might limit the amount of sodium available for exchange with potassium ions in the distal tubule (5), the presumed site of potassium secretion.

However, balance studies in patients with heart failure or cirrhosis and in dogs depleted of sodium by peritoneal dialysis have failed to demonstrate net potassium retention at a time when the urinary sodium excretion was very low (6, 7). Furthermore, when potassium salts were administered to these subjects, the potassium balance was not altered although significant hyperkalemia was observed. The elevation of plasma potassium was considerably greater than any change seen in similarly treated normal individuals. In these studies the hyperkalemia could not be definitely related to diminished renal excretion of potassium.

The present experiments were undertaken in an attempt to examine the capacity of the sodium depleted dog to excrete potassium during acute loading experiments and to test the hypothesis that the hyperkalemia observed in sodium depleted states might be the result of failure of the renal secretory mechanism.

## METHODS

Clearance studies were carried out in nine trained, unanesthetized female mongrel dogs, weighing 12 to 21 kilograms. Each dog was studied before and again after sodium depletion. The first study was done after the dogs had been maintained on a stock diet containing at least 100 mEq. of sodium per day and a supplement of 150 mEq. of potassium. Sodium chloride depletion of the animals was accomplished by peritoneal dialysis according to the method of Darrow and Yannet (8). The dogs were then maintained on a synthetic diet containing no sodium and 150 millimoles of potassium chloride per day. The animals were restudied two to seven days after the dialysis.

Renal clearances were performed using the clearance of exogenous creatinine as a measure of the glomerular filtration rate. Following a priming dose of creatinine and a half-hour equilibration period, two 20 minute control periods were obtained before either KCl or $K_2SO_4$ (potassium concentration 150 mEq. per L.) in 1 per cent creatinine solution was infused at a constant rate. Urine was collected from an indwelling catheter, and each clearance period was terminated by washing the bladder once with 15 ml. of distilled water. Venous blood samples were collected in heparinized syringes at the midpoint of each period.

Creatinine was measured in sodium tungstate filtrates of plasma and in diluted urine by the alkaline-picrate method. Sodium and potassium in diluted plasma and urine were determined by internal standard flame photometry. Chloride was estimated by a modification of the Volhard titration method. The calculation of the potassium clearance incorporated a ratio of ultrafiltrate concentration to plasma water concentration of 0.95 (Gibbs-Donnan effect).

[1] This work was supported in part by a grant from the United States Public Health Service, National Heart Institute (USPHS H-1275), and the Fleitas Foundation Fund.

## RESULTS

Table I summarizes 18 experiments in nine animals. The first five clearance studies (Dogs P, T, G, Sp and C) were carried out using KCl as the loading salt. In the remainder, $K_2SO_4$ was employed. The total amount of potassium administered, the potassium excreted and the sodium excreted in each clearance period is recorded. If the two studies in the same animal are compared it can be seen that each dog excreted approximately the same fraction of the administered potassium before and after sodium depletion. The average glomerular filtration rate following sodium depletion in these animals tended to be lower than in the control experiments. Appreciable reduction in plasma sodium was uniformly produced by the dialysis procedure, and urinary excretion of sodium was markedly reduced in all instances. These changes indicate that the amount of sodium in the tubular urine was considerably decreased in the studies done after dialysis. The plasma chloride concentration in the normal animals averaged 108 mEq. per L. before KCl was given. At the end of the infusion a maximum level of 117 mEq. per L. was observed. After depletion the average initial chloride concentration was 90 mEq. per L., and reached a maximum of 105 mEq. per L. after infusion of chloride salt.

The details of a representative experiment in which KCl was infused are given in Table II. Large amounts of potassium appeared in the urine both before and after sodium depletion, and the ratios of the clearance of potassium to the clearance of creatinine ($C_K/C_{Cr}$) were similar in the two situations. Following sodium depletion only small amounts of this ion were present in the bladder urine, and a marked increase in the ratio urine [K] to urine [Na] was apparent.

The results of a typical experiment in which $K_2SO_4$ was given are shown in Table III. Urine potassium content was higher in this control study than in that of Dog P above, and this increase could not be entirely accounted for in terms of the total dose administered in the two studies (*cf.*, Table I), but was consistent with the results one might expect when nonreabsorbable $SO_4^=$ is em-

TABLE I

*Summary of paired clearance studies in nine dogs before and after sodium chloride depletion* *

| Dog | Average creatinine clearance | | Plasma concentrations | | | | Total potassium infused | | Cumulative potassium excreted | | Potassium excreted (% of potassium infused) | | Cumulative sodium excreted | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Sodium | | Potassium | | | | | | | | | |
| | B‡ | A | B | A | B | A | B | A | B | A | B | A | B | A |
| | *ml./min.* | | *mEq./L.* | | | | *mEq.* | | *mEq.* | | | | *mEq.* | |
| P† | 67 | 44 | 148 | 132 | 3.4–5.6 | 5.7–9.5 | 93 | 105 | 34.3 | 46.4 | 36.5 | 43.0 | 9.4 | 0.7 |
| T | 80 | 73 | 144 | 129 | 4.2–7.0 | 5.0–7.6 | 125 | 117 | 45.6 | 51.6 | 30.4 | 43.0 | 12.6 | 1.2 |
| G† | 61 | 61 | 144 | 125 | 4.3–6.8 | 6.1–8.1 | 84 | 89 | 39.2 | 38.0 | 46.6 | 43.0 | 6.2 | 0.5 |
| Sp† | 40 | 35 | 146 | 135 | 4.1–6.0 | 4.6–8.8 | 52 | 68 | 8.9 | 13.1 | 17.2 | 19.3 | 3.5 | 0.4 |
| C | 69 | 55 | 142 | 132 | 4.6–7.0 | 5.6–6.7 | 124 | 90 | 68.0 | 45.9 | 54.8 | 51.0 | 15.9 | 0.4 |
| B | 63 | 34 | 145 | 124 | 3.7–5.2 | 4.6–8.0 | 103 | 75 | 58.5 | 41.6 | 57.0 | 55.5 | 35.2 | 1.7 |
| M | 72 | 46 | 146 | 119 | 4.5–5.8 | 5.7–6.1 | 88 | 48 | 32.0 | 33.5 | 36.8 | 70.0 | 9.7 | 0.5 |
| W | 33 | 40 | 145 | 136 | 3.9–7.0 | 4.7–7.7 | 81 | 91 | 25.0 | 52.5 | 30.9 | 57.5 | 10.7 | 3.5 |
| S† | 45 | 29 | 148 | 136 | 4.4–7.2 | 4.3–8.1 | 100 | 107 | 58.5 | 44.5 | 58.5 | 41.5 | 30.1 | 1.3 |

* The experimental protocol described (see Methods) was followed for each clearance study. KCl infusions were administered to Dogs P, T, G, Sp and C. The others received $K_2SO_4$. The plasma sodium concentrations recorded are those from blood drawn before the infusions were started. The range of plasma potassium concentrations from the beginning of each study to the end of the potassium infusion is shown. The total amount of potassium administered, the total amount of potassium excreted, and the total amount of sodium excreted during the potassium infusions are recorded.

† Died at conclusion of clearance study following Na depletion.

‡ B, before NaCl depletion; A, after NaCl depletion.

TABLE II

*Sodium, potassium and chloride excretion during potassium chloride infusion*

| Time | Plasma | | | Dog P, 17.3 Kg. Urine | | | Creatinine clearance | $C_K/C_{Cr}$* | Urine $\frac{[K]}{[Na]}$† |
|---|---|---|---|---|---|---|---|---|---|
| | Na | K | Cl | Na | K | Cl | | | |
| *min.* | *mEq./L.* | | | *μEq./min.* | | | *ml./min.* | | |
| | | | | Before NaCl depletion | | | | | |
| −88 | 148 | 3.4 | 108 | | | | | | |
| −60 to −45 | | | | Infuse 100 ml. of 3.5% creatinine I.V. | | | | | |
| −45 | | | | Infusion slowed to 1.5 ml./min. | | | | | |
| 0–20 | 144 | 3.5 | 106 | 34.0 | 6.8 | 27.8 | 52 | 0.04 | 0.20 |
| 20–50 | 146 | 3.4 | 107 | 12.7 | 3.2 | 19.1 | 64 | 0.02 | 0.25 |
| 51 | | | | Infuse KCl, 150 mEq./L. in 1% creatinine, 4.0 ml./min. | | | | | |
| 50–70 | 141 | 3.2 | 106 | 20.7 | 2.3 | 24.9 | 56 | 0.01 | 0.11 |
| 70–90 | 138 | 3.3 | 108 | 56.7 | 48.0 | 92 | 70 | 0.22 | 0.85 |
| 90–110 | 138 | 4.2 | 110 | 90.7 | 215.0 | 285 | 74 | 0.73 | 2.38 |
| 110–130 | 140 | 4.8 | 112 | 104 | 332.0 | 418 | 75 | 0.97 | 3.20 |
| 130–150 | 138 | 5.6 | 108 | 124 | 357.0 | 447 | 81 | 0.83 | 2.88 |
| 150 | | | | Infusion slowed to 3.5 ml./min. | | | | | |
| 150–170 | 138 | 5.4 | 103 | 53 | 325 | 376 | 67 | 0.94 | 6.15 |
| 170–190 | 137 | 5.5 | 113 | 15.9 | 278 | 283 | 70 | 0.76 | 17.5 |
| 190–210 | 137 | 5.3 | 112 | 4.7 | 156 | 132 | 63 | 0.49 | 33.2 |
| | | | | After NaCl depletion | | | | | |
| −60 | 132 | 5.7 | 99 | | | | | | |
| −45 to −30 | | | | Infuse 100 ml. of 3.5% creatinine I.V. | | | | | |
| −30 | | | | Infusion slowed to 1.5 ml./min. | | | | | |
| 0–20 | 126 | 5.5 | 94 | 5.2 | 173 | 291 | 62 | 0.53 | 32.3 |
| 20–40 | 130 | 5.2 | 94 | 4.9 | 113 | 165 | 47 | 0.52 | 23.1 |
| 41 | | | | Infuse KCl, 150 mEq./L. in 1% creatinine, 4.4 ml./min. | | | | | |
| 40–60 | 130 | 4.8 | 95 | 3.8 | 83 | 119 | 42 | 0.43 | 21.8 |
| 60–80 | 125 | 5.5 | 96 | 3.0 | 217 | 210 | 43 | 0.96 | 72.5 |
| 80–100 | 125 | 6.2 | 99 | 5.0 | 360 | 336 | 50 | 1.22 | 72.0 |
| 100–120 | 125 | 6.3 | 101 | 4.8 | 386 | 352 | 49 | 1.39 | 80.5 |
| 120–140 | 125 | 7.4 | 101 | 5.2 | 352 | 323 | 43 | 1.16 | 69.5 |
| 140–160 | 128 | 7.8 | 103 | 5.7 | 400 | 365 | 46 | 1.17 | 70.2 |
| 160–180 | 131 | 8.3 | 105 | 4.7 | 362 | 316 | 41 | 1.12 | 77.2 |
| 180–200 | 128 | 9.5 | 106 | 1.3 | 153 | 130 | 21 | 0.80 | 117.7 |

* $C_K$, clearance of K, ml./min.; $C_{Cr}$, clearance of creatinine, ml./min.; $C_K/C_{Cr}$, excreted K/filtered K.
† [ ] = concentration.

ployed (9). Sodium depletion produced a marked decrease in the amount of sodium in the urine, but large amounts of potassium again appeared in the urine during the clearance study despite a reduced dose of $K_2SO_4$. The $C_K/C_{Cr}$ ratios were actually higher after sodium depletion in this animal.

Three studies were accomplished in Dog W (Table IV). The first two were the standard control and postdialysis studies. The third was done following surgical constriction of the thoracic vena cava and the development, subsequently, of ascites. After operation and for six weeks before the clearance study, the 24 hour urine sodium from this animal was less than 2 mEq. per day. Potassium excretion appeared equally efficient in the control, in the sodium depleted, and in the sodium retaining states.

Figure 1 is a graphic representation of the amount of potassium excreted per minute during each 20 minute clearance period in the 18 clearance studies. The data in the left of Figure 1 were taken from experiments done in normal animals and those in the right side of the figure from the same animals following sodium depletion. The distribution of points in the two graphs is similar. The plasma potassium concentrations during the infusions have been averaged for each period and are also illustrated. In the studies done after sodium depletion the plasma potassium levels were higher than in the control studies.

TABLE III

*Sodium and potassium excretion during potassium sulfate infusion*

| Time | Plasma | | Dog B, 13 Kg. Urine | | Creatinine clearance | $C_K/C_{Cr}$* | Urine $\frac{[K]}{[Na]}$* |
|---|---|---|---|---|---|---|---|
| | Na | K | Na | K | | | |
| *min.* | *mEq./L.* | | *μEq./min.* | | *ml./min.* | | |
| | | | Before NaCl depletion | | | | |
| −60 | 145 | 3.7 | | | | | |
| −45 to −30 | | | Infuse 100 ml. 3.5% creatinine I.V. | | | | |
| −30 | | | Infusion slowed to 1.5 ml./min. | | | | |
| 0–20 | 139 | 3.7 | 14.9 | 65.6 | 57 | 0.33 | 4.4 |
| 20–40 | 139 | 3.1 | 33.0 | 41.6 | 56 | 0.25 | 1.3 |
| 41 | | | Infuse $K_2SO_4$, 150 mEq./L. in 1% creatinine, 3.8 ml./min. | | | | |
| 40–60 | 141 | 3.4 | 98.0 | 67.0 | 56 | 0.39 | 0.7 |
| 60–80 | 139 | 4.5 | 159.5 | 218 | 66 | 0.77 | 1.4 |
| 80–100 | 141 | 4.7 | 187 | 288 | 63 | 0.79 | 1.5 |
| 100–120 | 142 | 4.9 | 274 | 400 | 72 | 1.19 | 1.5 |
| 120–140 | 140 | 5.0 | 244 | 336 | 59 | 1.20 | 1.4 |
| 140–160 | 139 | 5.3 | 254 | 377 | 64 | 1.17 | 1.5 |
| 160–180 | 135 | 5.2 | 210 | 384 | 57 | 1.36 | 1.8 |
| 180–200 | 134 | 5.3 | 167 | 372 | 60 | 1.23 | 2.2 |
| 200–220 | 134 | 5.2 | 183 | 412 | 70 | 1.19 | 2.2 |
| | | | After NaCl depletion | | | | |
| −60 | 124 | 4.6 | | | | | |
| −45 to −30 | | | Infuse 100 ml. 3.5% creatinine I.V. | | | | |
| −30 | | | Infusion slowed to 1.5 ml./min. | | | | |
| 0–20 | 121 | 3.8 | 17.3 | 92.4 | 43 | 0.59 | 5.3 |
| 20–40 | 123 | 3.6 | 10.7 | 57.9 | 37 | 0.46 | 5.4 |
| 50 | | | Infuse $K_2SO_4$, 150 mEq./L. in 1% creatinine, 3.0 ml./min. | | | | |
| 50–70 | 125 | 4.0 | 3.2 | 44.4 | 39 | 0.30 | 13.9 |
| 70–90 | 123 | 4.6 | 3.8 | 153 | 40 | 0.87 | 40.3 |
| 90–110 | 123 | 5.6 | 7.1 | 235 | 33 | 1.16 | 33.1 |
| 110–130 | 123 | 5.7 | 10.5 | 238 | 28 | 1.56 | 22.6 |
| 130–150 | 122 | 6.3 | 15.0 | 299 | 33 | 1.51 | 20.0 |
| 150–170 | 122 | 6.4 | 6.6 | 220 | 29 | 1.25 | 33.4 |
| 170–190 | 121 | 8.0 | 16.8 | 305 | 24 | 1.67 | 18.2 |
| | | | Infusion slowed to 2 ml./min. | | | | |
| 190–210 | 121 | 7.4 | 13.2 | 326 | 29 | 1.60 | 24.6 |
| 210–230 | 121 | 7.1 | 6.8 | 258 | 33 | 1.16 | 37.9 |

* See Table II for meaning of these abbreviations.

In Figure 2 the cumulative excretion of potassium before and after sodium depletion is related to the concurrent cumulative excretion of sodium. It can be seen that the excretion of potassium remained high despite a marked reduction in urinary sodium and a diminution in glomerular filtration rate.

Figure 3 illustrates two examples of the hyperkalemia usually observed in the sodium depleted animal. The plasma potassium concentration has been correlated with the cumulative positive potassium balance. It is again evident that the higher plasma potassium concentration in the sodium depleted animal cannot be attributed to a difference in the amount excreted. Not only was the initial plasma potassium concentration of the sodium depleted dogs uniformly higher than that of the normal animals (*cf.*, Table I) but the difference in concentration was exaggerated as the potassium salts were infused. In each dog identical degrees of potassium retention were observed before and after sodium depletion despite marked differences in the levels of plasma potassium.

## DISCUSSION

The experiments reported here demonstrate that the renal excretion of infused potassium salt may

TABLE IV

*Sodium and potassium excretion during potassium sulfate infusion*

| Time | Plasma Na | Plasma K | Urine Na | Urine K | Creatinine clearance | $C_K/C_{Cr}$* | Urine $\frac{[K]}{[Na]}$* |
|---|---|---|---|---|---|---|---|
| *min.* | *mEq./L.* | | *μEq./min.* | | *ml./min.* | | |
| | | | Dog W, 12.3 Kg. | | | | |
| | | | Before NaCl depletion | | | | |
| −60 | 145 | 3.9 | | | | | |
| −45 to −30 | | | Infuse 100 ml. of 3.5% creatinine I.V. | | | | |
| −30 | | | Infusion slowed to 1.5 ml./min. | | | | |
| 0–20 | 144 | 2.9 | 133 | 55 | 41 | 0.48 | 0.41 |
| 20–40 | 139 | 2.8 | 128 | 60 | 39 | 0.58 | 0.47 |
| 41 | | | Infuse $K_2SO_4$, 150 mEq./L. in 1% creatinine, 3.0 ml./min. | | | | |
| 40–60 | 141 | 3.0 | 109 | 75 | 45 | 0.58 | 0.69 |
| 60–80 | 143 | 3.3 | 80 | 112 | 37 | 1.02 | 1.42 |
| 80–100 | 148 | 4.2 | 25 | 177 | 35 | 1.26 | 7.1 |
| 100–120 | | | 31 | 181 | | | 5.8 |
| 120–140 | | | 42 | 210 | | | 5.8 |
| 140–160 | | | 86 | 228 | | | 2.7 |
| 160–180 | | | 63 | 232 | 30 | | 3.7 |
| 180–200 | 141 | 5.2 | 52 | 234 | 27 | 1.75 | 4.5 |
| 200–220 | | 7.1 | 61 | 282 | 23 | 1.82 | 4.6 |
| | | | After NaCl depletion | | | | |
| −60 | 136 | 4.7 | | | | | |
| −45 to −30 | | | Infuse 100 ml. of 3.5% creatinine I.V. | | | | |
| −30 | | | Infusion slowed to 1.5 ml./min. | | | | |
| 0–20 | 130 | 3.5 | 1.1 | 65 | 39 | 0.50 | 59.0 |
| 20–40 | 136 | 3.5 | 1.9 | 51 | 31 | 0.49 | 26.8 |
| 42 | | | Infuse $K_2SO_4$, 150 mEq./L. in 1% creatinine, 3.4 ml./min. | | | | |
| 40–60 | 133 | 3.5 | 5.8 | 64 | 36 | 0.53 | 11.0 |
| 60–80 | 135 | 4.5 | 20.0 | 159 | 37 | 1.00 | 8.0 |
| 80–100 | 134 | 5.3 | 17.8 | 250 | 38 | 1.30 | 14.1 |
| 100–120 | 136 | 5.0 | 28.8 | 297 | 41 | 1.52 | 10.3 |
| 120–140 | 135 | 5.4 | 22.4 | 305 | 38 | 1.56 | 13.6 |
| 140–160 | 135 | 5.8 | 26.2 | 382 | 40 | 1.72 | 14.6 |
| 160–180 | 133 | 6.0 | 20.6 | 344 | 34 | 1.77 | 16.7 |
| 180–200 | 133 | 6.1 | 14.3 | 350 | 42 | 1.44 | 24.4 |
| 200–220 | 133 | 7.7 | 16.9 | 475 | 51 | 1.27 | 28.1 |
| | | | After development of ascites, dog on high NaCl intake | | | | |
| −60 | 143 | 5.1 | | | | | |
| −45 to −30 | | | Infuse 100 ml. of 3.5% creatinine I.V. | | | | |
| −30 | | | Infusion slowed to 1.5 ml./min. | | | | |
| 0–20 | 139 | 4.3 | 3.1 | 39.4 | 35 | 0.27 | 12.7 |
| 20–40 | 142 | 4.1 | 2.4 | 26.2 | 35 | 0.19 | 11.0 |
| 41 | | | Infuse $K_2SO_4$, 150 mEq./L. in 1% creatinine, 3.4 ml./min. | | | | |
| 40–60 | 143 | 4.2 | 4.7 | 46.9 | 31 | 0.38 | 9.9 |
| 60–80 | 146 | 3.9 | | | | | |
| 80–100 | 148 | 4.8 | 12.0 | 218.4 | 30 | 1.59 | 18.2 |
| 100–120 | 145 | 5.4 | 5.7 | 235.8 | 27 | 1.70 | 15.1 |
| 120–140 | 147 | 5.8 | 8.7 | 244.0 | 27 | 1.63 | 27.2 |
| 140–160 | | 5.6 | 15.5 | 271.9 | 22 | 2.32 | 18.1 |

* See Table II for meaning of these abbreviations.

not be demonstrably impaired by the imposition of a rather severe state of sodium chloride depletion. This depletion was characterized by a significant reduction in plasma sodium concentration, and frequently by a reduced glomerular filtration rate. There was, consequently, a considerable reduction in the filtered load of sodium, and the sodium concentration of the urine was extremely low. The degree of sodium depletion thus accomplished was, in general, greater than that ob-

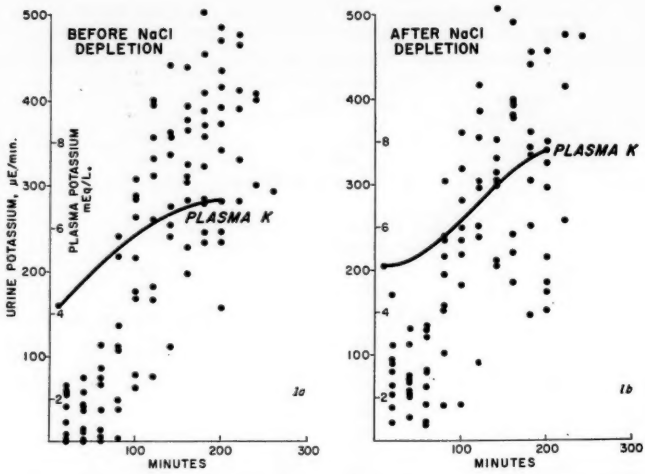



FIG. 1. POTASSIUM EXCRETION DURING EACH CLEARANCE PERIOD FOR NINE DOGS BEFORE AND AFTER SODIUM DEPLETION

The average plasma potassium concentration is represented by the heavy line. In the studies done in normal dogs the average rate of infusion of potassium was 537 μEq. per minute; in studies in sodium depleted animals it was 531 μEq. per minute.

served in patients. Therefore, it may be worthwhile to reappraise the hypothesis that potassium secretion is impaired in those situations in which the sodium content of urine in the distal tubule is reduced.

Demonstration of potassium secretion (as evidenced by a $C_K/C_{Cr}$ ratio greater than one) was facilitated by the reduction in GFR usually seen in sodium depleted dogs. Not only was secretion demonstrated, but the depleted animals were capable of excreting a fraction of the administered potassium which was equal to or greater than that in the control experiment. Moreover, there is considerable evidence that in the normal animal nearly all urinary potassium is secreted (5). The means by which an acute potassium load is excreted by the kidney of the salt depleted animal has not been fully elucidated. At least four possible explanations may be presented:

1. A large portion of the acute potassium load may be filtered and excreted without reabsorption. Such an assumption implies the abrupt development of faulty reabsorption. It is clear from the chronic experiment (7) and the data in the control periods before potassium infusion that considerable reabsorption of potassium does occur. However, the possibility remains that the

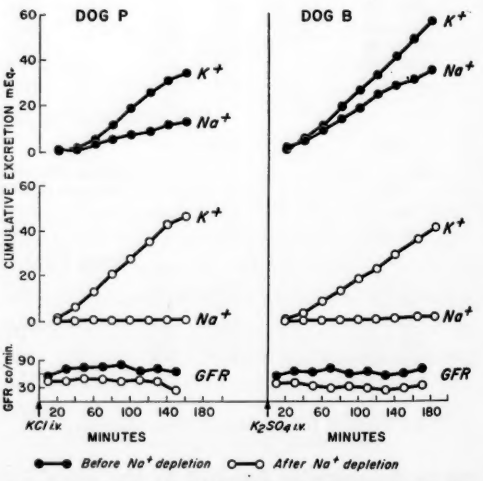



FIG. 2. CUMULATIVE POTASSIUM EXCRETION AFTER SODIUM DEPLETION

Results are similar to the control study, although urine sodium is markedly reduced and the glomerular filtration rate is lower.

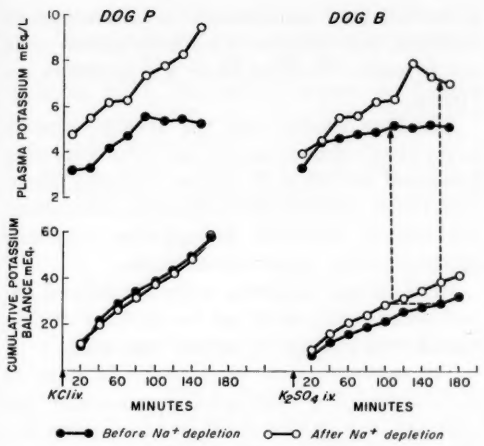



FIG. 3. RELATIONSHIP OF PLASMA POTASSIUM TO THE CUMULATIVE POTASSIUM BALANCE DURING POTASSIUM INFUSIONS BEFORE AND AFTER SODIUM CHLORIDE DEPLETION

After depletion there is a considerable hyperkalemia at a time when the cumulative potassium balance is similar to the control. The dotted lines indicate the differences in plasma potassium in the normal and sodium depleted states at comparable degrees of positive potassium balance.

reabsorptive capacity is limited and that this limit has been exceeded by the load.

2. It is also possible that some fraction of the administered potassium is secreted by the tubule with an anion. Present data cannot exclude this.

3. Proximal sodium reabsorption may be reduced when a large load of potassium chloride or sulfate is given. This would increase the amount of sodium available in the distal tubule for ion exchange with potassium. The slight increase in final urine sodium usually observed during potassium infusion could be interpreted as support for the suggestion that there was a change in proximal sodium reabsorption. If proximal reabsorption were constant one might expect a decrease in urine sodium excretion as potassium excretion increases.

4. Presently available evidence (10) suggests that approximately one-eighth of the filtered load of sodium would be available for potassium exchange in the distal tubular urine. If this concept is applied to states of sodium depletion there would be an adequate amount of sodium in the distal tubule even under the most severe conditions of sodium depletion. The experiments reported here do not exclude the possibility that potassium excretion has been accomplished by means of exchange of potassium ions from the tubule cell for sodium ions in the urine of the tubule. Indeed, the ion exchange mechanism may be operating maximally with the result that only a small amount of sodium escapes into the final urine.

The demonstration, under the conditions of the present study, that potassium excretion is not limited by sodium depletion, appears to be an unexpected observation in the light of the hypothesis advanced by Berliner (5) and Howell and Davis (4). These workers postulated that, if the amount of sodium available in the distal tubule is reduced either by a low sodium diet or the production of a sodium retaining state, potassium secretion by ion exchange for sodium is curtailed. Examples of the many instances which support this hypothesis have already been cited. However, in view of the findings reported here, factors (in addition to the available sodium) which might be concerned with the regulation of potassium secretion must be considered. The interrelationship of potassium and hydrogen is well known, and a competition between these two ions for renal secretion has been proposed (11). In the previously described situations in which limitation of potassium excretion was associated with sodium deprivation, there has been a tendency toward extracellular alkalosis. In extracellular alkalosis, an increased hydrogen ion concentration of renal tubular cells has been found (12). This probably favors Na-H exchange when the filtered load of sodium is low. When more sodium is available, greater stress may be applied to the mechanism and potassium secretion occurs by means of Na-K exchange. Operation of this competitive mechanism may be important in the potentiation of potassium excretion when there is abundant sodium in the distal tubule.

In contrast, animals receiving potassium chloride or sulfate infusions exhibit extracellular acidosis (9). The present data indicate that in both the normal and sodium depleted dogs a large amount of the infused potassium enters cells. The extracellular acidosis may be the result of the exchange of administered potassium for intracellular hydrogen and a corresponding intracellular alkalosis may result. One might postulate that in

the normal and the sodium depleted animals in the study reported here, a decrease in intracellular hydrogen and an increase in intracellular potassium favor secretion of this latter ion. A quantification of acid-base balance under the conditions of these experiments may be useful.

In the present study significant hyperkalemia was observed in all of the sodium depleted dogs during the infusion of potassium. The elevations of plasma potassium could not be accounted for by failure of renal excretion. Approximately 40 per cent of the administered dose appeared in the urine during these experiments. The remainder was stored temporarily outside the extracellular fluid, presumably within cells. The data suggest that the hyperkalemia of sodium depletion might be related to a change in the capacity to transfer additional potassium into cells. Sodium depletion may alter membrane transport of potassium or may actually lead to a change in intracellular environment which limits further storage. Yannet and Darrow (13) have found increased intracellular potassium in cats depleted of sodium chloride by peritoneal dialysis. This increase may be the cause of the reduction in the capacity for additional cellular uptake.

The study emphasizes again the hazards of potassium administration when there is sodium depletion. Following potassium infusions to sodium depleted animals, arrhythmias and electrocardiographic changes were frequently observed. Four of the nine animals reported here (P, G, Sp and S) died after the experiment. The net positive potassium balance in these animals was no greater following sodium depletion than in the control study, but at the conclusion of the potassium infusions (after Na depletion), the plasma potassium concentrations were all above 8 mEq. per L. In the sodium depleted state the cardiac toxicity appears to be directly related to the concentration of potassium in the extracellular fluid.

## SUMMARY

The effect of sodium depletion on plasma potassium concentration and on the capacity of the kidney to excrete potassium has been examined.

The renal excretion of potassium in sodium depleted dogs was quantitatively similar to that of the control animal. Renal tubular secretion of potassium was demonstrated in severe sodium depletion, and reduction of available sodium could not be made a limiting factor for potassium excretion.

The data suggest that the available sodium in the distal tubule is not the sole factor governing potassium excretion in sodium retaining states. It has been proposed that a change in intracellular pH may be important in regulating potassium secretion under these circumstances.

After sodium depletion animals exhibited hyperkalemia which could not be attributed to failure of renal excretion of infused potassium.

The cardiac toxicity of potassium in states of sodium depletion was again demonstrated and appeared to be related to an increased extracellular potassium concentration.

## ACKNOWLEDGMENT

The authors wish to thank Dr. John V. Taggart for help, advice and encouragement throughout the course of these studies.

## REFERENCES

1. Seldin, D. W., Welt, L. G., and Cort, J. The effect of pituitary and adrenal hormones on the metabolism and excretion of potassium (abstract). J. clin. Invest. 1951, **30**, 673.
2. Relman, A. S., and Schwartz, W. B. The effect of DOCA on electrolyte balance in normal man and its relation to sodium chloride intake. Yale J. Biol. Med. 1952, **24**, 540.
3. Milne, M. D., Hughes Jones, N. C., and Evans, B. M. Electrolyte excretion in states of potassium depletion in man *in* Ciba Foundation Symposium on the Kidney. Boston, Little Brown & Co., 1954, p. 212.
4. Howell, D. S., and Davis, J. O. Relationship of sodium retention to potassium excretion by the kidney during administration of desoxycorticosterone acetate to dogs. Amer. J. Physiol. 1954, **179**, 359.
5. Berliner, R. W. The kidney. Ann. Rev. Physiol. 1954, **16**, 269.
6. Laragh, J. H. The effect of potassium chloride on hyponatremia. J. clin. Invest. 1954, **33**, 807.
7. Laragh, J. H., and Capeci, N. E. Effect of administration of potassium chloride on serum sodium and potassium concentration. Amer. J. Physiol. 1955, **180**, 539.
8. Darrow, D. C., and Yannet, H. The changes in the distribution of body water accompanying increase and decrease in extracellular electrolyte. J. clin. Invest. 1935, **14**, 266.

9. Berliner, R. W., Kennedy, T. J., Jr., and Hilton, J. G. Renal mechanisms for excretion of potassium. Amer. J. Physiol. 1950, **162**, 348.
10. Smith, H. W. The Kidney: Structure and Function in Health and Disease. New York, Oxford University Press, 1951.
11. Berliner, R. W., Kennedy, T. J., Jr., and Orloff, J. Relationship between acidification of the urine and potassium metabolism. Amer. J. Med. 1951, **11**, 274.
12. Anderson, H. M., and Mudge, G. H. The effect of potassium on intracellular bicarbonate in slices of kidney cortex. J. clin. Invest. 1955, **34**, 1691.
13. Yannet, H., and Darrow, D. C. The effect of depletion of extracellular electrolytes on the chemical composition of skeletal muscle, liver, and cardiac muscle. J. biol. Chem. 1940, **134**, 721.

## ANNOUNCEMENTS OF MEETINGS

The Fiftieth Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Monday, May 5, 1958, at 9:00 A.M. at the Casino Theater on the Steel Pier.

The Fifteenth Annual Meeting of THE AMERICAN FEDERATION FOR CLINICAL RESEARCH will be held in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Sunday, May 4, 1958, at 9:00 A.M.

THE ASSOCIATION OF AMERICAN PHYSICIANS will hold their Seventy-First Annual Meeting in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Tuesday, May 6, 1958, at 9:30 A.M. and in the Vernon Room, Chalfonte-Haddon Hall on Wednesday, May 7, 1958, at 9:30 A.M.

## SPECIAL NOTICE TO SUBSCRIBERS

Post Offices will no longer forward the Journal when you move.

Please notify The Journal of Clinical Investigation, Business Office, 333 Cedar Street, New Haven 11, Conn., at once when you have a change of address, and do not omit the zone number if there is one.

# STUDIES ON THE NEONATAL DEVELOPMENT OF THE GLUCURONIDE CONJUGATING SYSTEM [1, 2]

By AUDREY K. BROWN and WOLF W. ZUELZER with the assistance of HERBERT H. BURNETT

*(From The Child Research Center of Michigan, the Department of Pediatrics, Wayne State University College of Medicine, and The Children's Hospital of Michigan, Detroit, Mich.)*

(Submitted for publication August 15, 1957; accepted October 31, 1957)

In jaundice of the newborn, whether "physiologic" or related to hemolytic disease, the bilirubin is known to exhibit the so-called "indirect" (van den Bergh) reaction with diazotized sulfanilic acid. Recently it has been shown that this type of bilirubin is unconjugated bilirubin, in contrast to the water soluble, direct reacting type which is a conjugated form, bilirubin-glucuronide (1–3). Conjugation is believed to occur chiefly in the liver, and bilirubin is excreted in the bile as a glucuronide (4).

Recent studies have demonstrated that an enzyme in the microsomes of liver transfers glucuronic acid from uridine-diphosphate-glucuronic acid (UDPGA) to bilirubin (5–7), a mechanism similar to that previously described for the transfer of glucuronic acid to phenolic (8–11), carboxylic (12), and amine acceptors (13). The reaction would appear to proceed as indicated in the equation:

$$\text{UDPGA} + \underset{\text{(indirect)}}{\text{bilirubin}} \xrightarrow{\text{glucuronyl transferase}} \underset{\text{(direct)}}{\text{bilirubin-glucuronide}} + \text{UDP}$$

It seemed likely that the cause of the indirect hyperbilirubinemia of the newborn might be related to a defect in the ability of the neonatal liver to perform glucuronide conjugation. The reason for such a failure might involve a number of steps in the development of this mechanism (Figure 1). The aim of this study was to define the step or steps at which the synthesizing system might be at fault.

Two enzyme systems involved in glucuronide synthesis were investigated. Since glucuronyl transferase activity would be the ultimate determinant of glucuronide formation, it seemed logical to explore first the glucuronyl transferase activity in newborn liver. Inadequate transferase activity would explain the accumulation of unconjugated bilirubin in the first few days of life even from normal red cell degradation. Gradual development of this system would explain the self-limitation of jaundice of the newborn.

To test this hypothesis, glucuronide synthesis was studied using microsomal fractions from fetal, newborn and adult guinea pig livers as the source of glucuronyl transferase, and bilirubin, ortho-aminophenol and phenolphthalein as the glucuronyl acceptors. In this phase of the study, to isolate the possible defect, pure uridine-diphosphate-glucuronic acid (UDPGA) was added to all reaction mixtures in order to test transferase activity alone in the presence of adequate UDPGA. Thus, specific enzymatic function could be assayed.

In the second phase, study was made of the capacity of the newborn liver to supply its own UDPGA. To investigate the development of this step in glucuronide synthesis, the activity of the enzyme which catalyzes the two-step oxidation of uridine-diphosphate-glucose (UDPG) to uridine-diphosphate-glucuronic acid (UDPGA) (10) was assayed. This enzyme, UDPG dehydrogenase, was first purified from the soluble fraction of liver homogenate by Strominger, Kalckar, Axelrod, and Maxwell (10). Activity of this specific $DPN^+$ dependent dehydrogenase can be determined from the rate of $DPN^+$ reduction (14). In this phase of the study of the glucuronide conjugating ability of the newborn, a comparison of UDPG dehydrogenase activity in the soluble fraction of liver homogenate from fetal, newborn and adult guinea pigs was made.

[1] This investigation was supported in part by Public Health Service Grant RG 5105, and by grants from The Children's Leukemia Foundation of Michigan and the Cerebral Palsy Fund, Lambda Tau Delta, Detroit.

[2] Presented in part before The Society for Pediatric Research, 27th Annual Meeting, Carmel, Calif., June, 1957.

1. Uridine triphosphate + α-glucose-1-phosphate $\xrightleftharpoons{\text{uridyl transferase}}$ UDP glucose + pyrophosphate.

2. UDP glucose + 2 $DPN^+$ $\xrightarrow{\text{UDPG dehydrogenase}}$ UDP glucuronic acid + 2 DPNH + $2H^+$.

3. UDP glucuronic acid + ROH $\xrightarrow{\text{glucuronyl transferase}}$ R-O-glucuronide + UDP.

FIG. 1. ENZYMATIC STEPS IN GLUCURONIDE SYNTHESIS

## MATERIALS AND METHODS

### *1. Biochemical materials*

a. Uridine-diphosphate-glucuronic acid (UDPGA),[3] uridine-diphosphate-glucose (UDPG), and diphosphopyridine nucleotide ($DPN^+$), were obtained from the Sigma Chemical Co., St. Louis, Mo.

b. Crystalline indirect bilirubin was obtained from Hoffman-LaRoche and Co., Nutley, N. J.

c. Ortho-aminophenol-glucuronide was supplied by Dr. Julius Axelrod, National Institutes of Health, for a reference standard.

### *2. Preparation of enzyme fractions*

a. *Glucuronyl transferase.* Guinea pig fetuses were obtained by caesarean section at approximately four, seven, and eight weeks' gestation. Adult guinea pigs, as well as newborn guinea pigs which ranged in age from 1 to 21 days, were killed by a blow on the head and the livers were immediately excised, chilled and weighed. In a number of instances Sprague-Dawley rats were similarly utilized. The livers were placed in 10 volumes of chilled 0.9 per cent KCl and homogenized in the cold with a Teflon homogenizer. After initial centrifugation at 5,000 × G in an International refrigerated centrifuge to remove nuclei and cellular debris, the supernatant fraction was centrifuged at 80,000 × G for 30 minutes in a Spinco Model E analytical ultracentrifuge. The sediment containing microsomes and mitochondria was washed and resuspended in cold KCl and again centrifuged at 80,000 × G. The sediment was then suspended in phosphate buffer, pH 7.4. This was used as the enzyme fraction containing "glucuronyl transferase."

b. *UDPG dehydrogenase.* For assay of UDPG dehydrogenase activity, the soluble fraction of guinea pig liver was used and fractions of three degrees of purity were assayed. For initial studies, the particle-free supernatant after the initial centrifugation of liver homogenate at 80,000 × G was used as the source of UDPG dehydrogenase. For the next group of studies the enzyme fraction precipitated from homogenized liver between 50 and 70 per cent saturation with ammonium sulfate was prepared by the method outlined by Tomkins (15).

In later studies, to obtain greater activity, the technique of Strominger, Maxwell, Axelrod, and Kalckar (14) was employed. The enzyme fraction by this method was precipitated between 35 and 55 per cent saturation with ammonium sulfate and purified stepwise, except that the final adsorption and elution from calcium phosphate gel was not employed.

### *3. Assay of glucuronyl transferase activity*

a. *Conjugation of bilirubin.* As the glucuronyl acceptor, bilirubin was used initially, since this substance was of immediate interest. In subsequent experiments ortho-aminophenol and phenolphthalein were used as the aglycones to test the same enzyme. Conversion of these substances to the glucuronide can be quantitated more precisely than can the conversion of bilirubin to bilirubin-glucuronide *in vitro*. The reaction mixture for the *in vitro* conversion of bilirubin was prepared according to the method of Schmid, Hammaker, and Axelrod (5) and consisted of the washed microsomal fraction, Tris [tris(hydroxy-methyl) aminomethane] buffer, pure UDPGA, magnesium chloride, and crystalline bilirubin (exact amounts used are summarized in Table I). The bilirubin was dissolved in 0.05 N sodium carbonate immediately before adding it to the reaction mixture. The reaction mixture for the conversion of bilirubin was incubated at 37° C. for 15 minutes under nitrogen in order

TABLE I

*Assay systems for conjugation of aglycones*

| | Bilirubin* | Ortho-amino-phenol | Phenol-phthalein |
|---|---|---|---|
| Micromoles of substrate | 3 | 0.66 | 0.15 |
| Gm. wet wt. of liver from which microsomal fraction was derived | 4† | 0.2 | 0.2 |
| "Tris" buffer‡ | | | |
| μM | 500 | 100 | 100 |
| pH | 8.5 | 8.0 | 8.0 |
| UDPGA (μM) | 4.0 | 0.2 | 0.2 |
| $MgCl_2$ (μM) | 50 | 50 | § |
| $H_2O$ (ml.) | to 12.0 | to 0.8 | to 1.0 |
| Incubation (min. at 37° C.) | 15 (under nitrogen) | 30 (in air) | 30 (in air) |

* Hoffman-LaRoche crystalline bilirubin.

† In some instances only 2 Gm. of liver was available from fetal or newborn livers, in which cases controls of corresponding amounts of adult livers were used.

‡ Tris (hydroxymethyl) amino-methane.

§ Reaction proceeded adequately without $MgCl_2$.

[3] Pure UDPGA was first obtained through the kindness of Dr. Julius Axelrod at the National Institutes of Health, and subsequently through the generosity of the Sigma Chemical Co., St. Louis, Mo.

to avoid the rapid deterioration of conjugated bilirubin which occurs in air. The mixture was then treated with an excess of diazotized sulfanilic acid and ethanol to convert the bilirubin and the bilirubin-glucuronide to their azo-derivatives. The mixture was shaken with chloroform to remove the protein and the azo-pigments were extracted with butanol. They were then separated and identified by ascending chromatography, following the technique of Schmid (1). Azo-derivatives of direct reacting bilirubin from urine and serum of patients with obstructive jaundice, and of commercial (indirect) bilirubin, were chromatographed as standards for the identification of the two types of bilirubin. The azo-pigments of bilirubin were quantitated spectrophotometrically after elution from the paper.

b. *Conjugation of ortho-aminophenol and phenolphthalein.* The systems for the conjugation of ortho-aminophenol and phenolphthalein were essentially similar to that described for bilirubin. Details of the assay systems are summarized in Table I and are adapted from those previously described (5, 10).

Pure UDPGA was added to the reaction mixture to provide an excess, because of the possibility that there might be a defect in the production of this glucuronyl donor in the newborn.

*4. Quantitation of glucuronide formation*

Ortho-aminophenol-glucuronide was determined colorimetrically by the method of Levvy and Storey (16). Quantitation of the glucuronide was made possible by using ortho-aminophenol-glucuronide as the standard.

In order to measure the amount of phenolphthalein-glucuronide formed, the free dye concentration was determined before and after incubation by the method of Talalay, Fishman, and Huggins (17). The formation of the glucuronide was represented by the disappearance of free dye. To further demonstrate this, the free dye was extracted with ethyl ether after incubation and the remainder of the incubation mixture which contained phenolphthalein-glucuronide was hydrolyzed by beta-glucuronidase for one hour at 37° C. The amount of phenolphthalein liberated by this specific hydrolysis was then measured.

Glucuronic acid was determined by the carbazole method of Dische (18). Microsome nitrogen content was determined by the micro-Kjeldahl technique.

*5. Indirect assay of UDPG dehydrogenase*

In the initial step, the particle-free supernatant from liver homogenate, containing the enzyme UDPG dehydrogenase, was incubated with pure UDPG and $DPN^+$ to catalyze the formation of UDPGA, using the technique described by Strominger, Kalckar, Axelrod, and Maxwell (10). Then glucuronide synthesis was performed as described above, except that no extra UDPGA was added to the UDPGA generated in the initial step. Ortho-aminophenol was the glucuronyl acceptor used. In this group of experiments results were expressed simply as the increase in optical density at 540 mμ after the color reaction for ortho-aminophenol-glucuronide had taken place.

*6. Direct assay of UDPG dehydrogenase activity*

The UDPG dehydrogenase activity in the two prepared fractions from liver homogenate was measured according to the procedures of Strominger, Maxwell, Axelrod, and Kalckar (14), in which there is specific reduction of $DPN^+$ by UDPG. The assay system consisted of UDPG (0.2 μM), $DPN^+$ (0.5 μM) and 0.2 ml. of the enzyme fraction in 0.5 ml. of 0.1 M glycine buffer, pH 8.7. The reaction mixture was placed in a 2 ml. Corex cuvette, light path 0.5 cm. The optical density at 340 mμ was read in a Beckman D.U. spectrophotometer (1 unit of UDPG dehydrogenase equals an increase in optical density of 0.001 per minute). Readings were taken each minute for the first 10 minutes, then at 5 minute intervals for the next 20 minutes.

## RESULTS

*1. Glucuronyl transferase activity*

When the microsomal fraction from adult guinea pig liver was used as the source of glucuronyl transferase, bilirubin, in the presence of UDPGA, was conjugated as bilirubin-glucuronide. No bilirubin-glucuronide formation could be demonstrated when the reaction mixture was incubated with microsomes from fetal or newborn guinea pig liver. When no UDPGA was added, bilirubin-glucuronide formation did not take place either in the adult or newborn system. Figure 2 demonstrates the identification of the unconjugated and the conjugated bilirubins by paper chromatography. The faster component (Rf 0.47) is the azo-pigment of unconjugated bilirubin. Only in the adult system was the slower component (Rf 0.39) seen. The Rfs of these two pigments compared well with the values established in this laboratory for the azo-pigment of commercial (unconjugated bilirubin) (0.44 to 0.48) and for that of the azo-pigment of direct bilirubin obtained from urine and serum of patients with obstructive jaundice (0.36 to 0.40). The slow component was shown to be the azo-pigment of bilirubin-glucuronide by the method of Schmid (1). Each azo-pigment was eluted, the bilirubin measured spectrophotometrically, and then specific hydrolysis with beta-glucuronidase at 37° C., pH 6.2, was carried out. After hydrolysis, glucuronic acid was determined. No glucuronic acid was found in the fast component or in the commercial bilirubin used in these experiments. In the eluate from the slow

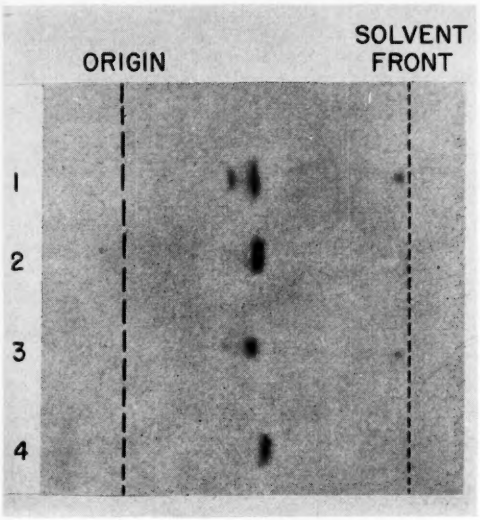



FIG. 2. RESULTS OF *IN VITRO* CONJUGATION OF BILIRUBIN

Paper chromatography demonstrating the separation of bilirubin and bilirubin-glucuronide after extraction of pigments from reaction mixtures. 1. Adult microsomal fraction (from 4 Gm. wet weight of liver), bilirubin, and UDPGA. 2. Adult microsomal fraction (from 4 Gm. wet weight of liver), bilirubin, and no UDPGA. 3. Adult microsomal fraction (from 2 Gm. wet weight of liver), bilirubin, and UDPGA. 4. Young (four days) microsomal fraction (from 2 Gm. wet weight of liver), bilirubin, and UDPGA.

Fast component indicates azo-pigment of unconjugated bilirubin. Slow component in 1 and 3 indicates azo-pigment of conjugated bilirubin. The faint (green) spot near the solvent front for 1 and 3 was presumably biliverdin.

moving spot, the molar relationship of the azo-pigment to glucuronic acid was found to be 1 : 1, indicating that bilirubin-glucuronide was present. Having demonstrated a qualitative defect in the ability of the fetal and neonatal liver to conjugate bilirubin, quantitative assays of glucuronyl transferase activity were undertaken using ortho-aminophenol and phenolphthalein as the aglycones.

Figure 3 shows a comparison of the amount of phenolphthalein conjugated when the microsomal fractions from 200 mg. of liver from fetal, newborn and adult guinea pigs were used as a source of glucuronyl transferase. The data in the figures represent the values for the disappearance of free phenolphthalein. In each instance an equal amount of phenolphthalein was recovered after beta-glucuronidase hydrolysis, indicating that the phenolphthalein had been conjugated in the reaction mixture. It is evident that at birth the guinea pig liver possesses only a slight degree of activity and that there is a gradual, steady increase in the ability to conjugate phenolphthalein during the postnatal period.

Entirely comparable results were obtained in corresponding experiments with ortho-aminophenol (Figure 4). In the fetus no activity was evident until close to term (about eight and one-half weeks in the guinea pig). To verify the apparent absence of this enzyme activity in the fetal liver, several experiments were performed in which the enzyme fraction from 1,000 mg. instead of 200 mg. of fetal liver was used. In some of the seven week fetuses there was a proportional increase in the amount of glucuronide formed, whereas the amount formed by the four week fetus was not affected (Table II).

In order to ascertain that the amounts of liver tissue used in the assays of fetal and adult mi-

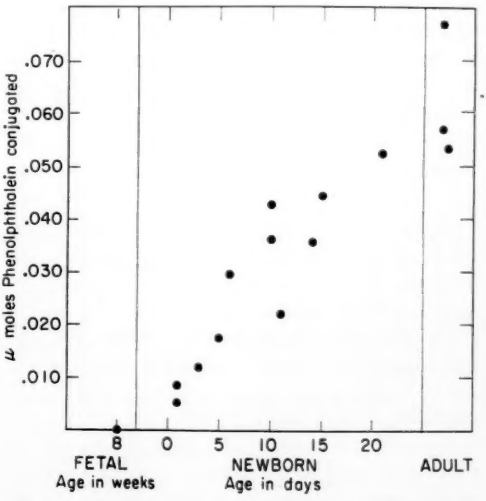



FIG. 3. COMPARISON OF THE AMOUNT OF PHENOLPHTHALEIN CONJUGATED BY FETAL, NEWBORN AND ADULT GUINEA PIG LIVER

Microsomal fraction from 200 mg. liver, with added UDPGA, incubated with 0.15 μM phenolphthalein for 30 minutes at 37° C.

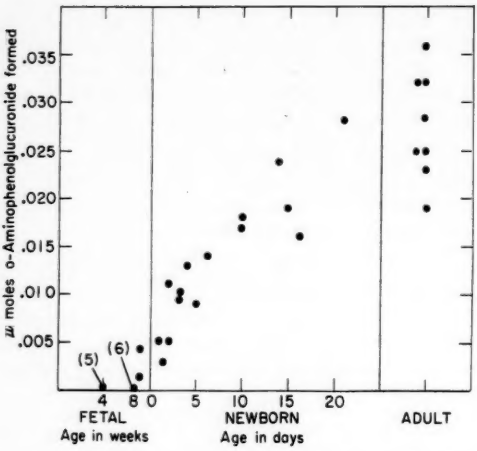



FIG. 4. COMPARISON OF THE AMOUNT OF ORTHO-AMINOPHENOL-GLUCURONIDE FORMED BY FETAL, NEWBORN AND ADULT GUINEA PIG LIVER

Microsomal fraction from 200 mg. liver, with added UDPGA, incubated with 0.66 μM ortho-aminophenol, for 30 minutes at 37° C. The parenthetical numbers 5 and 6 represent numbers of experiments performed at four and eight weeks.

crosomal activity were comparable, nitrogen determinations were performed on some of the washed microsomal preparations. The nitrogen content of the enzyme fractions are given in Table III.

### 2. *Indirect assay of UDPG dehydrogenase activity*

When soluble fraction of liver was used as the source of UDPG dehydrogenase in a system that included UDPG, $DPN^+$, and liver microsomal fractions, with ortho-aminophenol as the glucuronyl acceptor, glucuronide synthesis occurred. After incubation of the mixture for 30 minutes at 37° C., protein was removed and the color reaction for ortho-aminophenol-glucuronide was carried out. The ortho-aminophenol-glucuronide formation in this experiment is indicated only by the increase in transmittance at 540 mμ. Table IV summarizes the results of these experiments. It is known that synthesis of glucuronides in cell-free preparations of liver requires the presence of UDPGA (8, 9, 19). Since no UDPGA was added to the test system, glucuronic acid coupling

TABLE II

*Ortho-aminophenol conjugation with varying amounts of liver microsomes from fetal and adult guinea pigs*

| Age of animal | Weight of animal | Wet weight of whole liver | Ortho-aminophenol conjugated* (wet weight of liver used for microsomes) | |
|---|---|---|---|---|
| | | | 0.2 Gm. | 1.0 Gm. |
| | (*Gm.*) | (*Gm.*) | (*μM*) | (*μM*) |
| Adult | 1,074.0 | 44.6 | 0.020 | 0.079 |
| Adult | 845.0 | 39.7 | 0.030 | 0.096 |
| Fetus 7 weeks | 71.4 | 6.1 | 0 | 0.002 |
| Fetus 7 weeks | 97.4 | 6.8 | 0 | 0.002 |
| Fetus 7 weeks | 89.6 | 7.2 | 0 | 0 |
| Fetus 7 weeks | 47.6 | 3.5 | 0 | 0 |
| Fetus 4 weeks | 57.6 | 3.8 | 0 | 0 |
| Fetus 4 weeks | 68.2 | 3.0 | 0 | 0 |
| Fetus 4 weeks | 59.3 | 3.1 | 0 | 0 |

* Ortho-aminophenol-glucuronide obtained from Dr. Julius Axelrod was used as a reference standard.

TABLE III

*Nitrogen content of liver microsomal fraction in fetal, newborn and adult guinea pigs*

| Age of guinea pig | Nitrogen content of microsomal fractions* (*mg.*) |
|---|---|
| Fetus (weeks) | |
| 4 | 9.3 |
| 8 | 9.6 |
| 8 | 9.8 |
| Newborn (days) | |
| 1 | 9.7 |
| 2 | 9.5 |
| 3 | 9.2 |
| 4 | 8.7 |
| 5 | 10.0 |
| 10 | 9.4 |
| 10 | 9.8 |
| 11 | 9.6 |
| 11 | 9.3 |
| 13 | 9.4 |
| 14 | 9.1 |
| Adult | 10.5 |
| Adult | 10.0 |
| Adult | 10.7 |
| Adult | 10.2 |
| Adult | 10.3 |
| Adult | 10.0 |
| Adult | 10.2 |
| Adult | 9.7 |
| Adult | 10.2 |
| Adult | 10.1 |

* In each instance samples of microsomal fraction equivalent to 200 mg. wet weight of liver were taken.

under these circumstances could occur only if UDPGA were formed in the system by the action of UDPG dehydrogenase.

When both enzyme fractions, the glucuronyl transferase, and the UDPG dehydrogenase, used in the incubation system, were from adult liver, glucuronide synthesis proceeded readily when UDPG was also added, indicating that UDPG dehydrogenase had oxidized UDPG to UDPGA. Slight activity in the absence of UDPG probably represented the presence of some UDPG or UDPGA introduced into the system with the adult soluble fraction. By contrast, when a soluble fraction of newborn liver served as the source of the dehydrogenase, only minimal glucuronide synthesis proceeded even when the glucuronyl transferase was supplied by adult microsomes. Thus a marked decrease in newborn UDPG dehydrogenase as compared to that of the adult was demonstrated. As expected, when the newborn liver microsomal fraction was used as the source of glucuronyl transferase, there was virtually no demonstrable glucuronide synthesis, whether adult or newborn soluble fraction served as the source of dehydrogenase, indicating again the lack of transferase activity of newborn liver as previously shown.

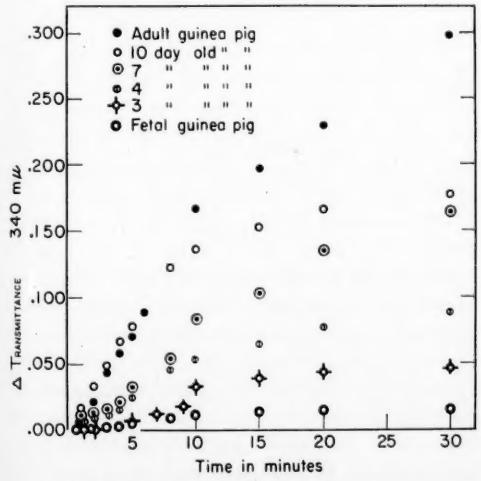



FIG. 5. REDUCTION OF DPN+ BY CRUDE PREPARATION OF UDPG DEHYDROGENASE IN SOLUBLE FRACTIONS FROM FETAL, NEWBORN AND ADULT GUINEA PIG LIVER

Activity during 30 minute period is indicated by change in optical density at 340 mμ.

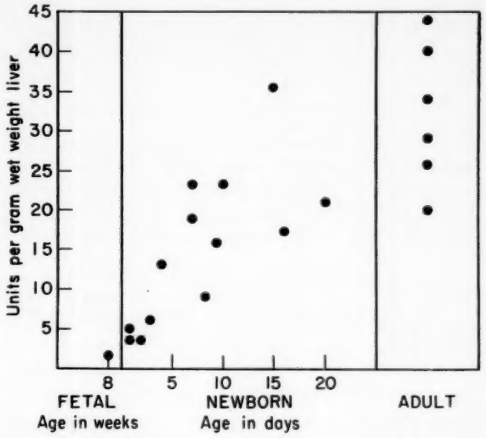



FIG. 6. COMPARISON OF UNITS OF UDPG DEHYDROGENASE ACTIVITY IN FETAL, NEWBORN AND ADULT GUINEA PIG LIVER

The enzyme fraction from liver homogenate precipitated between 50 and 70 per cent ammonium sulfate saturation was used. One unit of UDPG dehydrogenase activity equals change in transmittance at 340 mμ of 0.001 per minute.

### *3. Direct assay of UDPG dehydrogenase activity*

Direct measurements of the UDPG dehydrogenase activity, based on reduction of DPN+, were undertaken and confirmed the conclusions of the first experiments. Typical reaction curves of the dehydrogenase activity are illustrated in Figure 5.

UDPG dehydrogenase activity was virtually absent in the fetus. The same fraction from newborn livers showed very slight activity. Thereafter, as in the case of glucuronyl transferase, comparison of the enzymatic activity of different animals at various ages indicated the development of the enzyme system in the postnatal period. Levels of activity comparable to those found in the adult animal were evident by 15 to 20 days of age (Figure 6).

To evaluate further the quantitative difference between the adult and newborn enzyme activity, more purified preparations were made of the UDPG dehydrogenase contained in the soluble fraction of liver. When the fraction, precipitated between 35 and 55 per cent ammonium sulfate saturation, was used, the activity of the enzyme was markedly increased. When this more purified preparation of UDPG dehydrogenase was used,

TABLE IV

*UDPG dehydrogenase activity in adult and newborn guinea pig liver* *

| Composition of reaction mixtures† | Condition | Final transmittance‡ at 540 mμ |
|---|---|---|
| DPN + adult soluble fraction + adult microsomes | Incubated | 0.080 |
| UDPG + DPN + adult soluble fraction + adult microsomes | Not incubated | 0.015 |
| UDPG + DPN + adult soluble fraction + adult microsomes | Incubated | 0.418 |
| UDPG + DPN + newborn soluble fraction + adult microsomes | Incubated | 0.120 |
| UDPG + DPN + newborn soluble fraction + newborn microsomes | Incubated | 0.035 |
| UDPG + DPN + adult soluble fraction + newborn microsomes | Incubated | 0.040 |

* Using crude supernatant soluble fraction of liver homogenate.
† In addition to variables listed, all mixtures contained 0.66 μM ortho-aminophenol.
‡ Increase in transmittance at 540 mμ represents formation of ortho-aminophenol-glucuronide.

the activity (per unit weight of liver) in the two day old guinea pig liver was demonstrated to be about half that of the adult guinea pig liver (Table V).

TABLE V

*Activity of purified* UDPG dehydrogenase in adult and young guinea pig liver*

| Age of animal | UDPG dehydrogenase activity (*units/Gm. wet wt. liver*) |
|---|---|
| 2 days (pooled livers of 4 animals) | 156 |
| 6 days (pooled livers of 3 animals) | 231 |
| 12 days (pooled livers of 2 animals) | 306 |
| 25 days (pooled livers of 2 animals) | 330 |
| Adult | |
| Single animal | 318 |
| Single animal | 388 |

* Fraction precipitated between 35 and 55 per cent ammonium sulfate saturation.

To make certain that we were dealing with an enzyme that specifically oxidizes UDPG to UDPGA, the product of oxidation was adsorbed on charcoal and eluted with 50 per cent ethanol according to the technique of Strominger, Kalckar, Axelrod, and Maxwell (10). A positive carbazole reaction was obtained with the eluted product, indicating that oxidation of UDPG to UDPGA had taken place by the action of UDPG dehydrogenase.

## DISCUSSION

The enzyme systems in the liver of mammals involved in the formation of glucuronides have been shown to be of importance in the *in vitro* transformation of bilirubin to the excretable form, bilirubin-glucuronide (5–7). If a similar mechanism exists in the human, a defect in the system in the newborn could be responsible for accumulation of unconjugated bilirubin in the plasma. We have demonstrated that in newborn guinea pigs and rats there is very limited activity in the enzyme responsible for the transfer of the glucuronide moiety to an acceptor. Results indicate a gradual increase in activity of this enzyme, glucuronyl transferase, during the neonatal period. This defect was shown qualitatively for formation of bilirubin-glucuronide and quantitatively for formation of ortho-aminophenol-glucuronide and phenolphthalein-glucuronide, using *in vitro* assay systems in the presence of excess UDPGA.

The fetal liver in mid-gestation possesses virtually no glucuronide conjugating activity; the newborn seems to have about one-fifth the activity of the adult (per unit weight of liver used). At about 15 to 20 days of life, the enzyme system has an activity comparable to that of the adult.

These findings could be explained by a gradual, perhaps "adaptive," development of the enzyme system or the gradual disappearance of tissue inhibitors of the enzyme. Though beta-glucuronidase activity in the liver of newborn animals is greater than in the adult (20), it is unlikely that hydrolysis of the formed glucuronides explains the findings, since it has been found in work with liver slices that even with inhibition of beta-glucuronidase by saccharic acid there is a discrepancy between the glucuronide synthesizing ability of adult and newborn animals (20).

The data further indicate that the enzyme UDPG dehydrogenase required in the synthesis

of the physiologic glucuronic acid donor (UDPGA) is deficient. This enzyme activity also increases rapidly in the newborn period in approximate parallelism with the glucuronyl transferase.

The apparent rapidity of development of these enzyme systems is a challenging finding and the factors influencing their activity deserve attention. It is probable that in the newborn, there are enzymatic defects at other intermediate levels linking glucuronide synthesis to glycogen metabolism. Investigation of these steps as well as of means of augmenting development of enzyme activity in the newborn is under way.

Data directly relating these findings to the problem in the human newborn are difficult to obtain; however, indirect evidence based on total glucuronide excretion, presented elsewhere (21), supports the concept that the human newborn has an ineffective mechanism for the conjugation of glucuronides. This inefficiency is likely to be based on an enzymatic defect like that demonstrated *in vitro* in young mammals. It is also known that sulfo-bromophthalein sodium (BSP) is poorly handled by the neonatal liver (22). Preliminary observations in this laboratory suggest that this may also be indicative of a defect in glucuronide synthesis in the newborn. Coupling with glucuronic acid is one of the major detoxification mechanisms used for exogenous substances (23), as well as for endogenous substances including corticosteroids (11). Apart from furnishing a likely explanation for hyperbilirubinemia of the newborn the inadequacy of this mechanism must be of fundamental importance in newborn physiology.

## SUMMARY

Defects have been demonstrated in two enzymatic steps in the glucuronide synthesizing system in the fetal and newborn guinea pig. The glucuronyl transferase activity as well as the UDPG dehydrogenase activity are markedly deficient in the fetus and gradually increase during the first few days of life.

These enzymes are important in the formation of glucuronides, including bilirubin-glucuronide. The data support the hypothesis that the excess of "indirect" reacting, unconjugated bilirubin, regularly found in the human neonate, is due to inadequate development of the glucuronide conjugating mechanism.

## ACKNOWLEDGMENTS

We are indebted to Drs. Rudi Schmid, Julius Axelrod, Kurt Isselbacher and Gordon Tomkins for their many helpful suggestions while this work was in progress, and to Mr. Dan Broida of the Sigma Chemical Co., for his help and generosity in supplying UDPG and UDPGA.

## REFERENCES

1. Schmid, R. Direct-reacting bilirubin, bilirubin glucuronide in serum, bile, and urine. Science 1956, **124**, 76.
2. Billing, B. H., and Lathe, G. H. The excretion of bilirubin as an ester glucuronide, giving the direct van den Bergh reaction. Biochem. J. 1956, **63**, 6P.
3. Talafant, E. On the nature of direct and indirect bilirubin. V. The presence of glucuronic acid in the direct bile pigment. Chem. Listy 1956, **50**, 1329.
4. Schmid, R. The conjugation of bilirubin with glucuronic acid. J. Lab. clin. Med. 1956, **48**, 940.
5. Schmid, R., Hammaker, L., and Axelrod, J. The enzymatic formation of bilirubin glucuronide. Arch. Biochem. 1957, **70**, 285.
6. Grodsky, G. M., and Carbone, J. V. The synthesis of bilirubin glucuronide by tissue homogenates. J. biol. Chem. 1957, **226**, 449.
7. Arias, I. M., and London, I. M. Bilirubin glucuronide formation in vitro; Demonstration of a defect in Gilbert's disease. Science 1957, **126**, 563.
8. Dutton, G. J., and Storey, I. D. E. Uridine compounds in glucuronic acid metabolism. I. The formation of glucuronides in liver suspensions. Biochem. J. 1954, **57**, 275.
9. Smith, E. E. B., and Mills, G. T. Uridine nucleotide compounds of liver. Biochim. biophys. Acta 1954, **13**, 386.
10. Strominger, J. L., Kalckar, H. M., Axelrod, J., and Maxwell, E. S. Enzymatic oxidation of uridine diphosphate glucose to uridine diphosphate glucuronic acid. J. Amer. chem. Soc. 1954, **76**, 6411.
11. Isselbacher, K. J., and Axelrod, J. Enzymatic formation of corticosteroid glucuronides. J. Amer. chem. Soc. 1955, **77**, 1070.
12. Dutton, G. J. Uridine-diphosphate-glucuronic acid and ester glucuronide synthesis. Biochem. J. 1955, **60**, xix.
13. Axelrod, J., Inscoe, J. K., and Tomkins, G. M. Enzymatic synthesis of N-glucuronic acid conjugates. Nature (London) 1957, **179**, 538.
14. Strominger, J. L., Maxwell, E. S., Axelrod, J., and Kalckar, H. M. Enzymatic formation of uridine diphosphoglucuronic acid. J. biol. Chem. 1957, **224**, 79.

15. Tompkins, G. M. The enzymatic reduction of $\Delta^4$-3-ketosteroids. J. biol. Chem. 1957, **225**, 13.
16. Levvy, G. A., and Storey, I. D. E. The measurement of glucuronide synthesis by tissue preparations. Biochem. J. 1949, **44**, 295.
17. Talalay, P., Fishman, W. H., and Huggins, C. Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. J. biol. Chem. 1946, **166**, 757.
18. Dische, Z. A new specific color reaction of hexuronic acid. J. biol. Chem. 1947, **167**, 189.
19. Dutton, G. J., and Storey, I. D. E. The isolation of a compound of uridine diphosphate and glucuronic acid from liver. Biochem. J. 1953, **53**, xxxvii.
20. Karunairatnam, M. C., Kerr, L. M. H., and Levvy, G. A. The glucuronide-synthesizing system in the mouse and its relationship to beta-glucuronidase. Biochem. J. 1949, **45**, 496.
21. Brown, A. Studies on the neonatal development of the glucuronide conjugating system. Amer. J. Dis. Child. 1957, **94**, 510.
22. Yudkin, S., and Gellis, S. S. Liver function in newborn infants with special reference to excretion of bromsulphalein. Arch. Dis. Childh. 1949, **24**, 12.
23. Williams, R. T. Detoxification Mechanisms; The Metabolism of Drugs and Allied Organic Compounds. New York, John Wiley and Sons, 1947.

# ADRENAL ESTROGENS IN PATIENTS WITH METASTATIC BREAST CANCER [1]

By CHARLES D. WEST,[2] BARBARA DAMAST, AND O. H. PEARSON

*(From the Division of Clinical Investigation, Sloan-Kettering Institute for Cancer Research, and the James Ewing Hospital, New York, N. Y.)*

(Submitted for publication June 11, 1957; accepted October 31, 1957)

Numerous studies in both patients and experimental animals have established that estrogens play an important role in the growth of some breast cancers. It is generally agreed that the beneficial effect of bilateral oophorectomy in some patients with metastatic breast cancer is due to the elimination of the principal source of estrogens. Inevitably, patients who obtain castration remissions relapse. Many will respond favorably to subsequent adrenalectomy or hypophysectomy (1–3). As one possible explanation for these observations, it has been suggested that the adrenal cortex might function as an extragonadal source of estrogens. Since certain adrenal cortical tumors cause feminization in men and since other hypercortical states are frequently associated with the excretion of excessive amounts of estrogens (4), it is conceivable that normal adrenal cortical tissue might function as a significant source of estrogens in patients with breast cancer. The demonstration of biologically active estrogens in the urine of ovariectomized women by Dao (5) and Smith and Emerson (6) supports this concept. That the pituitary gland might be involved in the production of adrenal estrogens is suggested by the work of Nathanson, Engel, and Kelley (and in the discussion of Reference 7 by Paschkis) who reported increased levels of urinary estrogens in patients given adrenocorticotrophic hormone (ACTH).

The purpose of this study was to investigate the possible role of adrenal estrogens in breast cancer by attempting to isolate and identify estrogens excreted by castrated patients and to determine the effects of adrenalectomy, hypophysectomy, and the administration of ACTH upon their excretion.

[1] This project was generously supported in part by a grant from the United States Public Health Service [C-2241 (C3)].

[2] Scholar of the American Cancer Society. Present Address: Veterans Administration Hospital, Salt Lake City, Utah.

## ANALYTICAL METHODS

The analytical methods used in this investigation have been reported in detail elsewhere (8). Urine specimens were hydrolyzed with $\beta$-glucuronidase and extracted continuously with ether for 48 hours. The phenolic fractions were prepared from the ether extracts by partitioning between toluene and 1 N NaOH. Unless otherwise specified, the phenolic fractions were routinely fractionated as outlined in Figure 1. Estrone, estradiol and estriol were separated from the phenolic fractions by multiple sequential fractionations by countercurrent distribution and paper chromatography.

The technique used for countercurrent distribution was developed by Craig and Craig (9) and adapted to the analysis of estrogens by Engel (10). Countercurrent distribution served not only for fractionation, but also to provide information on the identity, purity and quantity of the isolated estrogens.

The fundamental techniques used for paper chromatography were developed by Bush (11) and adapted to the analysis of estrogens by Mitchell and Davies (12). The solvent systems, time of development and temperature for the paper chromatographies are indicated in Figure 1. The estrogens on the paper chromatograms were visualized by staining with an aqueous solution of 1 per cent ferric chloride and 1 per cent potassium ferricyanide as described by Axelrod (13).

Estrone, estradiol and estriol were measured by photofluorometry using Engel's modification (10) of the method of Bates and Cohen (14). To obtain biological confirmation of the chemical methods, the isolated estrogens were assayed for estrogenic activity by either the rat uterine weight or the rat vaginal cornification methods (15, 16).

To support the identification of the free estrogens by paper chromatography and countercurrent distribution, the acetate derivatives were prepared and analyzed by paper chromatography against authentic estrogen acetates. Acetylation was carried out in acetic anhydride and pyridine overnight at room temperature.

## RESULTS

Urines from patients without ovaries were analyzed for estrogens by the methods described

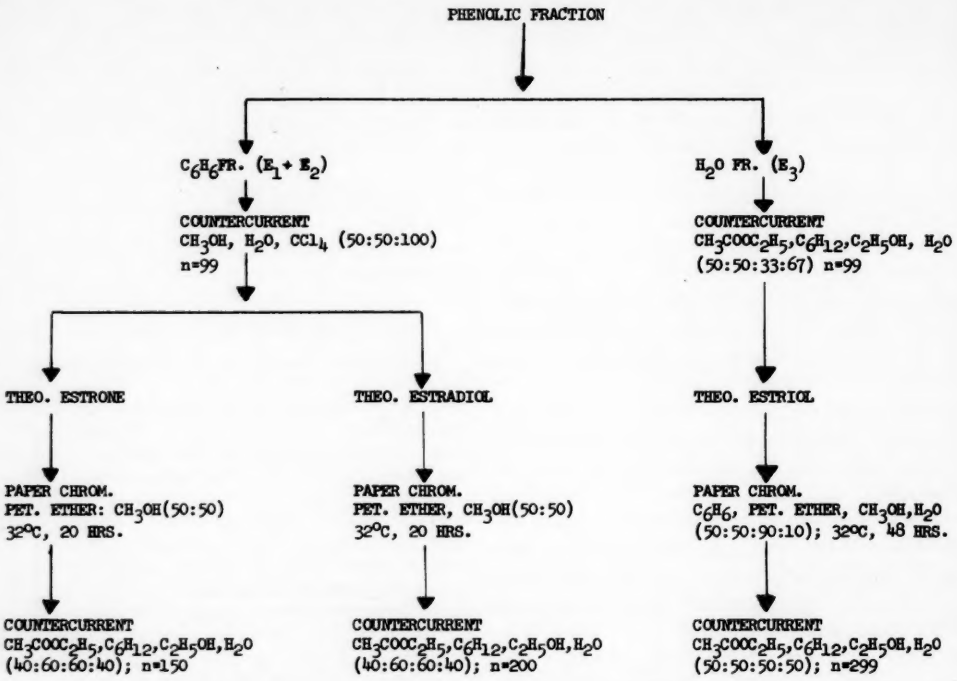



FIG. 1. OUTLINE OF METHOD OF FRACTIONATION FOR URINARY ESTROGENS

$E_1$, estrone; $E_2$, estradiol-17$\beta$; $E_3$, estriol. Solvent ratios are by volume; n equals number of transfers.

both during control periods and while the patients were on ACTH, in an attempt to increase the excretion of urinary estrogens by stimulating the adrenal cortex. The results obtained in one patient will be presented in detail to afford an opportunity for critical evaluation of the methods used.

This 66 year old patient (A.S.) had a radical mastectomy for carcinoma of the breast six years prior to this study, at which time she was 12 years postmenopausal. She developed osseous metastases two years later and was treated with both estrogens and androgens without improvement. In April, 1956, a hypophysectomy was performed and the patient had a remission lasting six months. Upon relapse, the metastatic osseous disease was treated with X-ray and a radiation dose of 2,000 r was delivered to both ovaries. The present estrogen excretion study was done three months later during the time the patient's neoplastic disease was advancing rapidly.

In order to stimulate the adrenal cortices of this hypophysectomized patient to maximal secretory activity, 50 mg. of ACTH was administered intramuscularly every six hours. After one week of ACTH therapy the adrenal cortices were actively secreting, as evidenced by a striking increase in urinary 17-ketosteroids and 17-hydroxycorticoids and the development of sodium retention and hypokalemic alkalosis. At this time a three day urine specimen was collected for estrogen analyses.

The phenolic fractions were prepared from the urine samples and analyzed for estrone, estradiol and estriol as outlined in Figure 1. The results of the initial countercurrent distribution of the benzene soluble material in the phenolic fraction are shown in Figure 2A. The distribution curve exhibited two nonhomogeneous peaks composed of mixtures of fluorescent phenols. It was theoretically possible that estrone could have been one of the fluorescent phenols making up the first

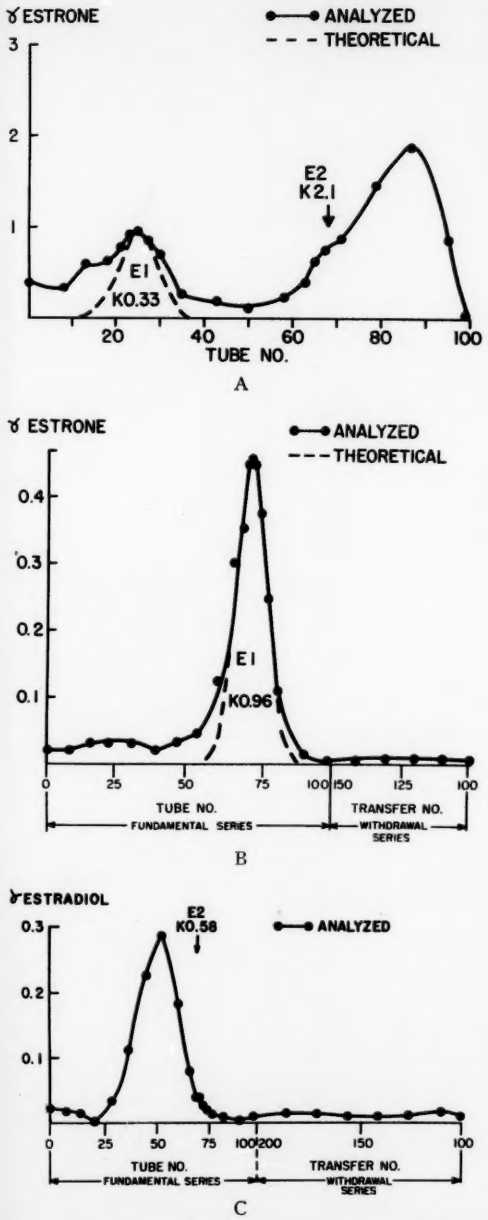



FIG. 2. COUNTERCURRENT DISTRIBUTION CURVES FOR ESTRONE ($E_1$) AND ESTRADIOL ($E_2$) ON PATIENT A.S.

●——●, analyzed curves; – – – –, theoretical curves. A. Initial countercurrent distribution of the benzene fraction of the phenolic extract in $CH_3OH$, $H_2O$, $CCl_4$ (50:50:100); n equals 99; partition coefficient (K) for $E_1$ equals 0.33; $K_{E_2}$ equals 2.1.

B. Countercurrent distribution for estrone following paper chromatography in $CH_3COOC_2H_5$, $C_6H_{12}$, $C_2H_5OH$, $H_2O$ (40:60:60:40); n equals 150; $K_{E_1}$ equals 0.96.

C. Countercurrent distribution for estradiol following paper chromatography in $CH_3COOC_2H_5$, $C_6H_{12}$, $C_2H_5OH$, $H_2O$ (40:60:60:40); n equals 200; $K_{E_2}$ equals 0.58. Single withdrawal procedure used in B and C.

peak, and estradiol one of those in the more polar peak.

To explore these possibilities further the tubes which would theoretically have contained estrone were combined and chromatographed on paper as indicated in Figure 1. The fraction from the paper chromatogram corresponding in mobility to reference standards of authentic estrone was eluted and analyzed by countercurrent distribution for a second time in a different solvent system. This time a single fluorescent compound was obtained, and the distribution curve agreed with the theoretical curve for estrone (Figure 2B). The 24 hour excretion of estrone was calculated from this countercurrent distribution curve to be 10 micrograms.

An aliquot of this material was analyzed by paper chromatography for the second time. As can be seen by the photograph of the finished paper chromatogram (Figure 3), the isolated material had the same mobility as the two reference standards of authentic estrone on each side.

The acetate derivative of the isolated material was prepared and analyzed simultaneously with reference standards of estrone acetate and free estrone by paper chromatography in petroleum ether and methanol (1:1) at 24° C. The chromatographic behavior of the acetate derivative was identical with that of estrone acetate, providing additional evidence for the identification of the isolated material as estrone.

The tubes from the initial countercurrent distribution of the benzene fraction (Figure 2A), which theoretically would have contained estradiol, were combined and analyzed by paper chromatography and again by countercurrent distribution. The final distribution curve did not exhibit a peak in the region expected for estradiol. A more polar fluorescent phenolic compound was observed but unidentified (Figure 2C).

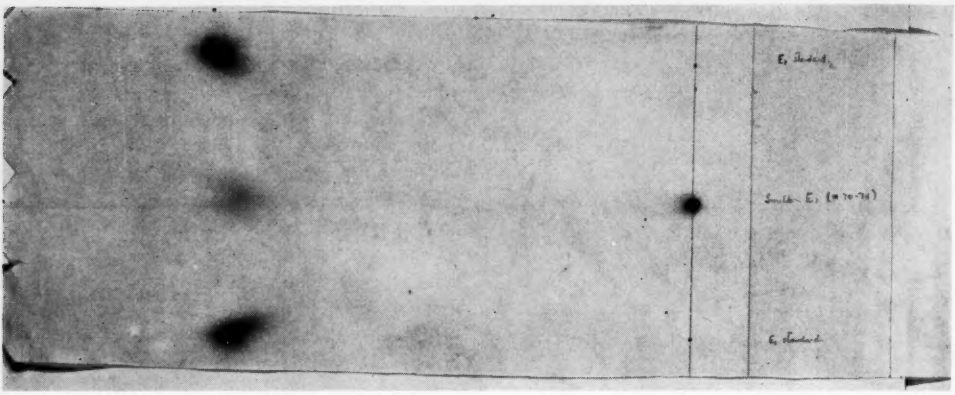


FIG. 3. PHOTOGRAPHS OF THE PAPER CHROMATOGRAM OBTAINED ON THE ESTRONE FRACTION FROM THE FINAL COUNTERCURRENT DISTRIBUTION, PATIENT A.S.

The middle spot is the isolated estrone with two reference standards of authentic estrone on each side. Developed in methanol: petroleum ether (1:1 v/v) for nineteen and one-half hours at 32° C.

An attempt to identify estriol was successful. The initial countercurrent distribution of the water soluble material in the phenolic fraction revealed a mixture of fluorescent phenols which could have contained estriol (Figure 4A). The tubes which theoretically would have contained any estriol present were combined, fractionated further by paper chromatography, and analyzed again by countercurrent distribution. This time a single fluorescent compound was obtained whose distribution curve fit the theoretical distribution curve for estriol exactly (Figure 4B). From these countercurrent data it was calculated that 51 micrograms of estriol was excreted daily by the patient.

One aliquot of the isolated material was again chromatographed on paper and identified as estriol (Figure 5A). A second aliquot was acetyl-

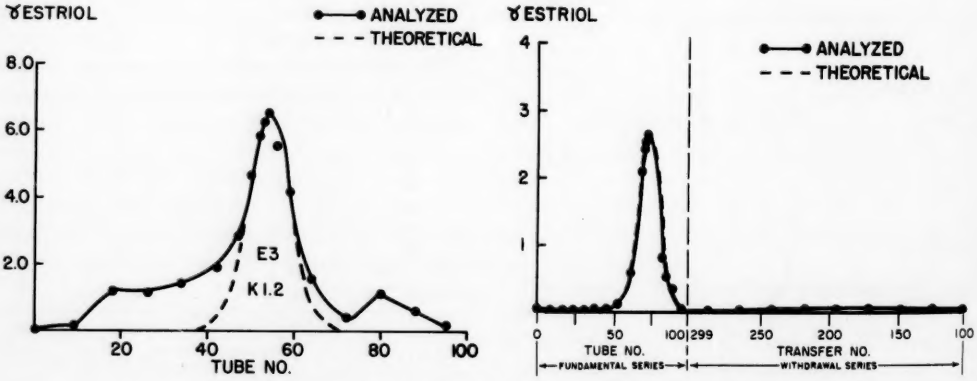
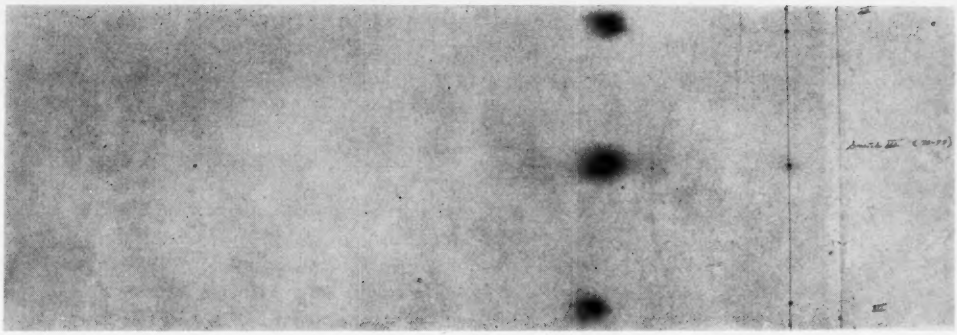


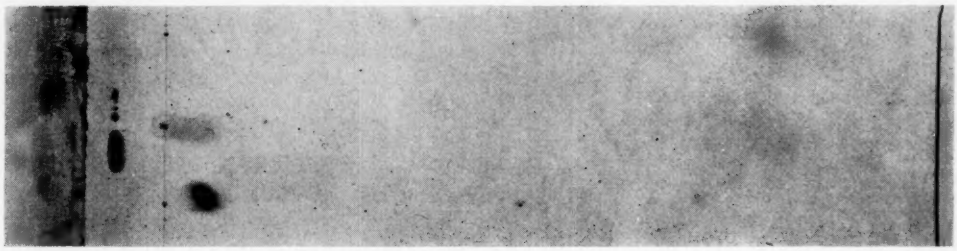

FIG. 4. COUNTERCURRENT DISTRIBUTION CURVES FOR ESTRIOL ($E_3$) ON PATIENT A.S.

A. Initial distribution of aqueous fraction of the phenolic extract in $CH_3COOC_2H_5$, $C_6H_{12}$, $C_2H_5OH$, $H_2O$ (50:50:33:67); n equals 99; $K_{E_3}$ equals 1.2.

B. Countercurrent distribution for estriol following paper chromatography in $CH_3COOC_2H_5$, $C_6H_{12}$, $C_2H_5OH$, $H_2O$ (50:50:50:50); n equals 299; $K_{E_3}$ equals 0.35. Single withdrawal procedure used.



A



B

FIG. 5. PHOTOGRAPHS OF PAPER CHROMATOGRAMS OBTAINED ON THE ESTRIOL FRACTION FROM THE FINAL COUNTERCURRENT DISTRIBUTION, PATIENT A.S.

A. Middle spot is the isolated estriol with estriol reference standards on each side, developed in petroleum ether, benzene, methanol, $H_2O$ (50:50:90:10) for 48 hours at 32° C.

B. Middle spot is the acetate derivative of the isolated estriol with reference standards of estriol triacetate and estriol on each side. Developed in methanol, $H_2O$, petroleum ether (80:20:100 v/v) for three hours at 32° C.

ated and identified as estriol triacetate by paper chromatography (Figure 5B).

A third aliquot was assayed quantitatively for estrogenic activity by the rat uterine weight method. Doses of 0.2, 0.4, and 1.0 microgram of isolated estriol, as determined fluorometrically, were injected into each of three castrated 21 day old female rats at each dose level, in six divided doses on three successive days. An estriol standard dose response curve was run simultaneously, as well as controls. The isolated material gave a positive bioassay equivalent to 41 micrograms of estriol excreted daily, in comparison with a value of 51 micrograms per 24 hours which was calculated from the final countercurrent distribution data, thus providing biological confirmation for the chemical identification.

Under the influence of ACTH administration the amounts of estrone and estriol excreted by this 66 year old patient (A.S.) were within normal limits for an actively menstruating woman. Perhaps of significance is the fact that she excreted no measurable amounts of estradiol. Since the patient was 12 years postmenopausal, had undergone X-ray castration and hypophysectomy, and excreted the estrogens under the influence of ACTH, it would appear highly probable that these urinary estrogens or their precursors originated from the adrenal cortex. Nevertheless, the ovaries cannot be absolutely ruled out in this patient as the site of origin, since they were still *in situ* and X-ray castration and hypophysectomy may not have destroyed their hormonal function.

Similar estrogen excretion studies were carried out in two other patients with metastatic breast cancer who had been surgically castrated (M.H. and E.Z.) and one other patient who had been castrated and hypophysectomized (M.G.), both

TABLE I

*Estrogen excretion by castrated patients with metastatic breast cancer and the effect of adrenocorticotrophic hormone (ACTH)* *

| Pt. | Age | Endocrine status | Control | | | ACTH treatment | | |
|---|---|---|---|---|---|---|---|---|
| | | | $E_1$ (μg./24 hrs.) | $E_2$ (μg./24 hrs.) | $E_3$ (μg./24 hrs.) | $E_1$ (μg./24 hrs.) | $E_2$ (μg./24 hrs.) | $E_3$ (μg./24 hrs.) |
| M. H. | 62 | Bilat. oophorect. | 2 | 0 | 1 | 19 | 0 | 38 (25)† |
| E. Z. | 42 | Bilat. oophorect. | 0 | 0 | 0 | 0 | 0 | 6 (3) |
| A. S. | 66 | Spontaneous menopause X-ray cast. Hypophysectomy | | | | 10 | 0 | 51 (41) |
| M. G. | 29 | Bilat. oophorect. Hypophysectomy | 0 | 0 | 0 | 0 | 0 | 5 (6) |
| M. M. | 43 | Bilat. oophorect. | 0 | 0 | 0 | | | |
| L. S. | 48 | Bilat. oophorect. | 0 | 0 | (35) | | | |
| C. M. | 35 | Bilat. oophorect. | 0 | 0 | (17) | | | |

* $E_1$, estrone; $E_2$, estradiol; $E_3$, estriol.
† Estrogen values in parentheses determined by bioassay and the other values by photofluorometry.

before and while on ACTH therapy. The results are summarized in Table I.

One castrated patient (M.H.) excreted 2 micrograms of estrone and 1 microgram of estriol daily during the control period. The estrone was isolated as a pure fluorescent compound by a combination of countercurrent distribution and paper chromatography and measured quantitatively by fluorometry as previously described. The isolated estrone gave a positive biological assay for estrogens by causing complete cornification of the vaginal epithelium in 15 out of 15 castrated adult female rats in doses of 0.035 microgram per rat administered intravaginally. Appropriate controls and standards were run simultaneously. The estriol was also isolated in a homogeneous fluorescent form and measured by fluorometry. It gave a positive test for estrogenic activity by the vaginal cornification method in doses of 0.03 microgram per rat.

With the administration of ACTH the excretion of estrone by patient M.H. increased from 2 to 19 micrograms per 24 hours; and estriol, from 1 to 38 micrograms per 24 hours.

None of the three classical estrogens could be identified in the urine of the other castrated patient (E.Z.) during the control period, but upon the administration of ACTH she excreted 6 micrograms of estriol daily. Neither estrone nor estradiol were found in the urine during the ACTH period.

No identifiable estrogens could be detected in the control urine of patient M.G. who had undergone bilateral oophorectomy and hypophysectomy. Upon the administration of ACTH, she excreted 5 micrograms of estriol daily, but no estrone or estradiol. The results obtained on patient A.S. which were presented above in detail are included in Table I.

None of the four castrated patients (M.H., E.Z., A.S. and M.G.) excreted detectable amounts of estradiol either during the control period or on ACTH. However, all of them excreted a fluorescent phenol which did not separate from estradiol on paper chromatography but did on subsequent countercurrent distribution. This compound is slightly more polar than estradiol and is so far unidentified.

Using a less rigorous method estrogen excretion studies were carried out in three additional ovariectomized patients with metastatic breast cancer (M.M., L.S. and C.M.). With this method estrone, estradiol and estriol were separated from each other by a single countercurrent

distribution of the phenolic fraction using 100 transfers in a solvent system made up of methanol, water, $CHCl_3$ and $CCl_4$ (70:30:40:60). If the distribution curve, as determined fluorometrically, was consistent with the presence of any of the three estrogens, the total amount of the individual estrogen was measured by assaying the appropriate peak tubes for estrogenic activity by the rat uterine weight method. Although estrone, estradiol and estriol can be separated from each other by the single countercurrent distribution used, they cannot be isolated in a fluorogenically homogeneous form and accurate quantitative measurement by fluorometry is impossible. In addition the three classical estrogens may not necessarily separate from other unknown estrogenic materials in the urinary extracts which would affect the results obtained by bioassay. Nevertheless, the results obtained by this less rigorous technique in the three castrated patients (C.M., M.M. and L.S.) are included in Table I to demonstrate further that patients without ovaries can excrete estrogens.

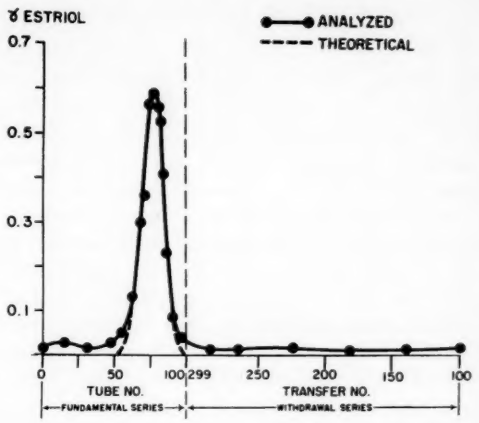



FIG. 6. COUNTERCURRENT DISTRIBUTION CURVE OBTAINED FOR ESTRIOL FOLLOWING AN INITIAL DISTRIBUTION AND PAPER CHROMATOGRAPHY IN A CASTRATED, ADRENALECTOMIZED, HYPOPHYSECTOMIZED PATIENT

●——●, analyzed curve; – – – –, theoretical curve for estriol; $K_{E_3}$ equals 0.35; n equals 299; solvent system, $CH_3COOC_2H_5$, $C_6H_{12}$, $C_2H_5OH$, $H_2O$ (50:50:50:50). Single withdrawal procedure used.

None of the three patients excreted estrone or estradiol in amounts which were detectable by countercurrent distribution. However, the distribution curves were compatible with the excretion of estriol in two of the patients (C.M. and L.S.). Therefore, the single peak tubes which theoretically contained the maximum amount of estriol were assayed by the rat uterine weight method. In this way it was determined that patient C.M. excreted 17 microgram equivalents of estriol in 24 hours; and patient L.S., 35 micrograms in 24 hours. The comparatively high values for estriol in these two patients could have been due to contaminating estrogenic materials which were not separated from estriol by the single countercurrent distribution.

No estrogens were demonstrated in the urine of the third castrated patient (M.M.) by this method. It may have been significant that this patient differed from all the rest of the castrated patients in that her neoplastic disease was in remission at the time the estrogen analyses were done.

The demonstration of estrone and estriol in the urine of castrated patients and the increased excretion upon ACTH administration strongly suggest that the adrenal cortex is involved in the production of these urinary estrogens or their precursors. If this interpretation is correct, patients without both ovaries and adrenals should not excrete estrogens.

To test this theory, urines from two castrated, adrenalectomized patients and two other patients who had been surgically castrated, adrenalectomized and hypophysectomized (for a total of four patients without either ovaries or adrenals) were analyzed for estrogens by the complete procedure outlined in Figure 1. None of these four patients excreted estrone or estradiol, and estriol could not be demonstrated in the urine of three of these patients. However good evidence for the excretion of estriol by the fourth patient, who had undergone bilateral oophorectomy, adrenalectomy, and hypophysectomy, was obtained. By the procedure outlined in Figure 1, a homogeneous fluorescent compound was isolated whose distribution curve coincided with the theoretical curve for estriol (Figure 6). The amount of estriol excreted daily was calculated from the final countercurrent distribution curve to be 11 micrograms. The acetate derivative of this material was prepared and identified as estriol triacetate by paper chromatography. In addition, it was shown that

the isolated material had estrogenic biological activity.

## DISCUSSION

In this investigation it was demonstrated that castrated patients with metastatic breast cancer may excrete significant amounts of estrone and estriol in their urine, but not estradiol. With the additional observation that the administration of ACTH increased excretion, it seems highly probable that these urinary estrogens or their precursors were produced by the adrenal cortex. The observation that patients without both ovaries and adrenals usually failed to excrete these estrogens lends support to this conclusion.

Whether the isolated urinary estrogens were actually secreted by the adrenal cortex or whether they represent metabolic products of some other adrenal precursor could not be ascertained from this study. There is indirect evidence to support both of these possibilities. With the isolation of estrone from adrenal cortical tissue by Beall (17) and the demonstration by Meyer (18) that 19-hydroxy-$\Delta^4$ androstene-3, 17-dione can be converted to estrone by adrenal tissue, it would appear that adrenal cortical tissue has the necessary enzyme systems for synthesizing estrogens and that secretion of estrogens by the adrenal gland is a real possibility. On the other hand, it has been demonstrated that certain $C_{19}$ neutral steroids of the androgenic series, some of which are produced by the adrenal cortex, are converted to estrogens both *in vivo* and *in vitro* by a variety of tissues (18–21). Under the conditions of the present experiments the adrenal cortex could have secreted these $C_{19}$ neutral steroids or other unknown precursors which were metabolized in part to estrogens elsewhere in the body and excreted in the urine.

The possibility that corticosteroids, such as cortisol and cortisone, might be converted in part to estrogens was considered since this conversion would play an important role in the management of adrenalectomized and hypophysectomized patients with estrogen-dependent breast cancer. Since the adrenalectomized and hypophysectomized patients in this study failed to excrete measurable amounts of estrogens even though they were receiving maintenance doses of cortisone throughout the study, it would appear that physiological levels of cortisone are not metabolized to estrogens to any great extent. To examine the possibility of this conversion more rigorously 600 mg. of cortisol was given intravenously to a castrated, adrenalectomized, hypophysectomized patient, and the urine analyzed for estrogens. Estrone, estradiol and estriol could not be demonstrated in the urine. These observations suggest that corticosteroids are not converted to estrogens in any significant amounts.

It is of interest that estriol was found more consistently in the urine of castrated patients than any other estrogen. Estriol is usually considered to be an estrogen metabolite rather than a secretory product. It cannot be ascertained from this study whether the estriol isolated from urine was a secretory product of the adrenal cortex or a metabolic end product of some other precursor.

The demonstration that the adrenal cortex in patients with metastaic breast cancer can be a source of significant amounts of estrogens is consistent with the theory that relapses following castration remissions are due to adrenal estrogens and that subsequent remissions following adrenalectomy are due to the removal of this source of estrogens, but does not prove it. Proof of this theory will depend upon whether the growth of the metastatic cancer in these patients can be correlated with the level of urinary estrogens during relapses and remissions.

The increased excretion of urinary estrogens by castrated patients upon the administration of ACTH and the absence of estrogens in the urine of castrated, hypophysectomized patients suggest that endogenous ACTH might be involved in the control of estrogen production by the adrenal cortex. If this is the case, one of the possible mechanisms by which hypophysectomy could cause remission in castrated patients is through secondary suppression of estrogen production by the adrenal gland.

In view of the fact that one of the castrated, adrenalectomized, hypophysectomized patients excreted appreciable amounts of estriol, other possible sources of estrogens besides the ovaries and adrenal glands, such as the diet or accessory ovarian or adrenal tissue, must be considered to explain relapses occurring in some patients with estrogen-dependent tumors.

SUMMARY

Significant amounts of estrone and estriol have been demonstrated in the urine of castrated patients with metastatic breast cancer by means of countercurrent distribution, paper chromatography, and bioassay methods. The excretion of these estrogens by castrated patients increased strikingly upon the administration of ACTH. With one exception, castrated and adrenalectomized patients failed to excrete identifiable amounts of estrogens. These observations suggest that estrogens or their precursors are produced by the adrenal cortex and that endogenous ACTH might be involved in the control of this estrogen production. Estriol was demonstrated in the urine of one patient who had been castrated, adrenalectomized, and hypophysectomized, raising the possibility of additional sources of estrogens besides the ovaries and adrenals, such as diet and accessory ovarian or adrenal tissue.

The possible pathophysiological significance of these findings in breast cancer was discussed.

ACKNOWLEDGMENT

The authors acknowledge with gratitude the help and advice given by Drs. T. F. Gallagher, W. O. Money, M. D. Lipsett and Rulon W. Rawson; and the technical assistance of Mrs. Louise Berman, Mr. Theodore Hall and Miss Dorothy Sellitto.

REFERENCES

1. Huggins, C., and Bergenstal, D. M. Influence of bilateral adrenalectomy, adrenocorticotrophin, and cortisone acetate on certain human tumors. Science 1951, **114**, 482.
2. Luft, R., and Olivercrona, H. Experiences with hypophysectomy in man. J. Neurosurg. 1953, **10**, 301.
3. Pearson, O. H., Ray, B. S., Harrold, C. C., West, C. D., Li, M. C., Maclean, J. P., and Lipsett, M. B. Hypophysectomy in the treatment of advanced cancer. Trans. Ass. Amer. Phycns 1955, **68**, 101.
4. Soffer, L. J. Diseases of the Adrenal, 2nd ed. Philadelphia, Lea and Febiger, 1948, pp. 249–252.
5. Dao, T. L. Y. Estrogen excretion in women with mammary cancer before and after adrenalectomy. Science 1953, **118**, 21.
6. Smith, O. W., and Emerson, K., Jr. Urinary estrogens and related compounds in postmenopausal women with mammary cancer: Effect of cortisone treatment. Proc. Soc. exp. Biol. (N. Y.) 1954, **85**, 264.
7. Nathanson, I. T., Engel, L. L., and Kelley, R. M. The effect of ACTH on the urinary excretion of steroids in neoplastic disease *in* Proc. Second Clinical ACTH Conf. New York, The Blakiston Co., 1951, vol. I, p. 54.
8. West, C. D., Damast, B., and Pearson, O. H. Urinary estrogen excretion in Cushing's syndrome. J. clin. Endocr. 1958, **18**, 28.
9. Craig, L. C., and Craig, D. Extraction and distribution *in* Technique of Organic Chemistry, A. Weissberger, Ed. New York, Interscience Publishers, Inc., 1950, vol. 3, p. 171.
10. Engel, L. L. The chemical estimation of steroid hormone metabolites *in* Recent Progr. Hormone Res. New York, Academic Press Inc., 1950, vol. 5, p. 335.
11. Bush, I. E. Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem. J. 1952, **50**, 370.
12. Mitchell, F. L., and Davies, R. E. The isolation and estimation of the steroid oestrogens in placental tissue. Biochem. J. 1954, **56**, 690.
13. Axelrod, L. R. The chromatographic fractionation and identification of compounds related to the estrogens *in* Recent Progr. Hormone Res. New York, Academic Press Inc., 1954, vol. 9, p. 69.
14. Bates, R. W., and Cohen, H. Experimental basis for selecting the optimal conditions for fluorometry of natural estrogens. Endocrinology 1950, **47**, 166.
15. Lauson, H. D., Heller, C. G., Golden, J. B., and Sevringhaus, E. L. The immature rat uterus in the assay of estrogenic substances, and a comparison of estradiol, estrone, and estriol. Endocrinology 1939, **24**, 35.
16. Emmens, C. S. Precursors of oestrogens. J. Endocr. 1941, **2**, 444.
17. Beall, D. The isolation of oestrone from ox adrenals. J. Endocr. 1940, **2**, 81.
18. Meyer, A. S. Conversion of 19-hydroxy-$\Delta^4$-androstene-3,17-dione to estrone by endocrine tissue. Biochim. biophys. Acta 1955, **17**, 441.
19. Heard, R. D. H., Jellinck, P. H., and O'Donnell, V. J. Biogenesis of estrogens: The conversion of testosterone-4-$C^{14}$ to estrone in the pregnant mare. Endocrinology 1955, **57**, 200.
20. West, C. D., Damast, B. L., and Pearson, O. H. The conversion of testosterone to estrogens in castrated, adrenalectomized human females. J. biol. Chem. 1956, **218**, 409.
21. Baggett, B., Engel, L. L., Savard, K., and Dorfman, R. I. The conversion of testosterone-3-$C^{14}$ to $C^{14}$ estradiol-17$\beta$ by human ovarian tissue. J. biol. Chem. 1956, **221**, 931.

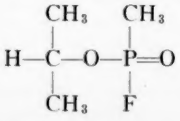
# EFFECTS IN MAN OF THE ANTICHOLINESTERASE COMPOUND SARIN (ISOPROPYL METHYL PHOSPHONOFLUORIDATE)[1]

By DAVID GROB AND JOHN C. HARVEY

*(From the Department of Medicine, The Johns Hopkins University and Hospital, Baltimore, Md.)*

(Submitted for publication August 6, 1957; accepted October 31, 1957)

A number of organic esters of phosphoric acid derivatives have been synthesized which have pharmacologic effects attributable to the inhibition of cholinesterase (ChE) enzymes. The effects resemble those produced by physostigmine and neostigmine (Prostigmin®) but are more intense and prolonged. Di-isopropyl fluorophosphate (DFP) has been studied in detail (1–4) and has been employed as a therapeutic agent in the management of abdominal distention (2), urinary retention, and glaucoma (5). Tetraethyl pyrophosphate (TEPP) (6) and octamethyl pyrophosphoramide (OMPA) (7) proved to be of some value in the management of myasthenia gravis, but have been replaced by less potent anticholinesterase compounds. Parathion, bis-(monoisopropylamino)-fluorophosphine oxide (Mipafox®), hexaethyl tetraphosphate (HETP), and TEPP have been widely used as agricultural insecticides, and their indiscriminate dispersal has resulted in a number of instances of poisoning, some of them fatal (8–10). The nerve gases are related compounds which, because of their high toxicity and physical properties, are among the most potent chemical warfare agents (11–14). One of the most important of these is sarin (isopropyl methyl phosphonofluoridate) (15), which has also been designated GB, and has the structural formula:



Another is tabun (dimethylamido-ethoxyphosphoryl cyanide) (16), which has been designated GA.

In the present study the effect of sarin on human ChE enzymes *in vitro* was compared with that of tabun, TEPP, DFP, neostigmine and parathion. Sarin was administered orally to 10 normal volunteer subjects and observations were made of signs and symptoms and of changes in plasma and red blood cell cholinesterase activity, electrical activity of the brain, movement of air into and out of the lungs, and response to atropine. The effects of the intra-arterial injection and conjunctival instillation of sarin were also investigated.

## METHODS

The liquid sarin employed was 90 per cent pure by chemical analysis (17), but since the compounds which constitute the remaining 10 per cent may have anticholinesterase activity, the material was regarded as 100 per cent sarin in all calculations. Solutions of sarin in water and in propylene glycol were administered daily over periods of one to four days. The concentration of these solutions ($4 \times 10^{-6}$ to $2.5 \times 10^{-4}$ M) was confirmed by colorimetric estimation, and by determination of inhibition of plasma and red blood cell cholinesterase *in vitro* and of toxicity to rats. Solutions were adjusted to a pH of 5 to 6, at which hydrolysis is slowest, and stored at 5° C. for up to three days. At the end of this time there was less than five per cent decrease in the active concentration of sarin in aqueous solution, and no change in propylene glycol solution.

The cholinesterase activity of plasma (heparinized) and red blood cells was determined in duplicate or triplicate by electrometric determination of change in hydrogen ion concentration (18) before and after the administration of sarin. The standard deviation of parallel replicate determinations was four per cent for plasma cholinesterase and three per cent for the red blood cell enzyme, and five per cent for replicate determinations on separate days following storage of frozen plasma or hemolyzed red blood cells.

For the studies of anticholinesterase activity of sarin and tabun carried out *in vitro*, plasma (heparinized) and red blood cells were obtained from normal subjects, and skeletal muscle and brain were obtained immediately post mortem from patients who had died of diseases that did not directly involve these tissues. Cholinesterase

[1] This work was supported by a contract between the Johns Hopkins University and the U. S. Army Chemical Corps, and was originally reported in Medical Division (Chemical Corps) Research Report Numbers 17 and 18, August, 1950.

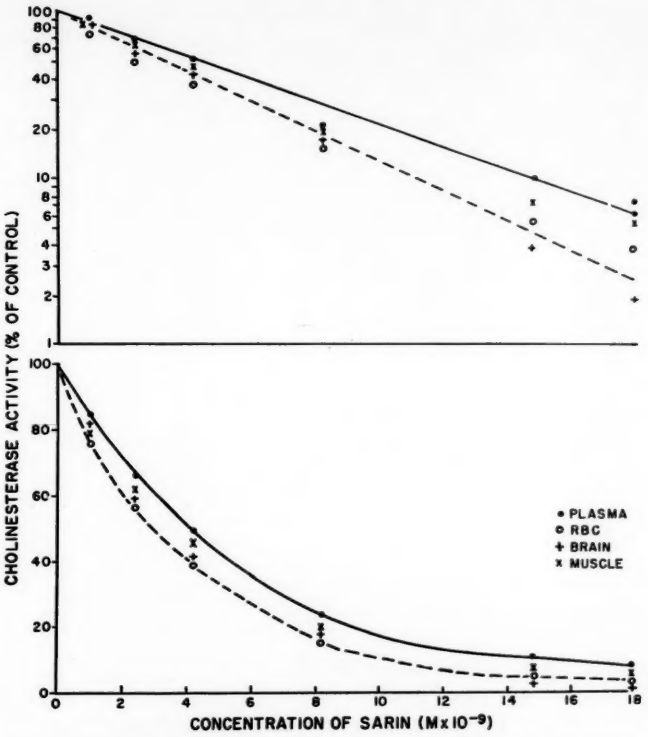



FIG. 1. EFFECT OF SARIN ON HUMAN PLASMA, RED BLOOD CELL, BRAIN, AND MUSCLE CHOLINESTERASE ACTIVITY *IN VITRO*

The same data are presented in the upper graph on a logarithmic scale, and in the lower graph on a linear scale. The concentrations of the enzyme preparations are recorded in Table I.

activity was determined in duplicate or triplicate manometrically (1). The standard deviation of parallel replicate determinations was five per cent.

## RESULTS

### *I. Effect of sarin on the cholinesterase activity of human plasma, red blood cells, muscle and brain in vitro*

#### *A. Inhibition of cholinesterase*

Plasma cholinesterase was slightly less sensitive to inhibition by sarin than the cholinesterases present in red blood cells, muscle and brain, which were approximately equally sensitive (Figure 1 and Table I). Increasing concentrations of sarin resulted in increasing inhibition of enzyme activity (Figure 1). Each increment in concentration inhibited the same *fraction* of cholinesterase and had a diminishing effect on the *amount* inhibited. The logarithm of the fraction of cholinesterase that remained active decreased linearly with increasing concentration of sarin, or, the logarithm of the reciprocal of the fraction that remained active was directly proportional to the concentration of sarin:

$$\log \frac{ChE_1}{ChE_0} = -k\,S, \quad \text{or,} \quad \log \frac{ChE_0}{ChE_1} = k\,S,$$

where $ChE_0$ equals cholinesterase activity just before the addition of sarin; $ChE_1$ equals cholinesterase activity one hour later; and S equals concentration of sarin. One hour after the addition

TABLE I

*The in vitro sensitivity of human plasma, red blood cell, brain and muscle cholinesterases to inhibition of sarin, tabun, TEPP, DFP, neostigmine and parathion* *

| | Plasma ChE | RBC ChE | Brain ChE | Muscle ChE |
|---|---|---|---|---|
| Sarin | $4.2 \times 10^{-9}$ | $3 \times 10^{-9}$ | $3.3 \times 10^{-9}$ | $3.6 \times 10^{-9}$ |
| Tabun | $1.3 \times 10^{-8}$ | $1.5 \times 10^{-8}$ | $1.5 \times 10^{-8}$ | $2 \times 10^{-8}$ |
| TEPP | $5 \times 10^{-9}$ | $3.5 \times 10^{-8}$ | $3.2 \times 10^{-8}$ | $3.5 \times 10^{-8}$ |
| DFP | $9.5 \times 10^{-9}$ | $4 \times 10^{-7}$ | $3 \times 10^{-7}$ | $2.5 \times 10^{-7}$ |
| Neostigmine | $4 \times 10^{-8}$ | $8 \times 10^{-7}$ | $4 \times 10^{-7}$ | $3 \times 10^{-7}$ |
| Parathion | $1 \times 10^{-6}$ | $1.2 \times 10^{-5}$ | $1.3 \times 10^{-5}$ | $1.5 \times 10^{-5}$ |

* The molar concentration of each anticholinesterase agent is recorded which produced 50 per cent inhibition of the ChE activity of a solution or homogenate which hydrolyzed $4.8 \times 10^{-4}$ mM acetylcholine bromide per hour in the absence of inhibitor. This rate of hydrolysis was produced by 51 mg. per ml. of plasma, 124 mg. per ml. of brain (cerebral cortex) homogenate, 58 mg. per ml. of muscle (psoas) homogenate, and 34 mg. per ml. of red blood cell contents. The initial concentration of acetylcholine bromide was 0.015 M. ChE and antiChE compounds were mixed 45 minutes before the addition of acetylcholine.

of sarin, cholinesterase could not be reactivated by standing, dilution or dialysis against running water.

### *B. Comparison with other anticholinesterase compounds*

The inhibitory activity of sarin against the cholinesterase enzymes of the red blood cells, muscle and brain was greater than that of the other anticholinesterase compounds, being approximately 5 times that of tabun, 10 times that of TEPP (6), 100 times that of DFP (1), and 4,000 times that of parathion (19) under the conditions of the experiment (Table I). The inhibitory activity of sarin against plasma cholinesterase was approximately equal to that of TEPP, and was greater than that of the other anticholinesterase compounds. The cholinesterases of red blood cells, muscle and brain were approximately equally sensitive to inhibition by each of the compounds studied. Plasma cholinesterase was slightly less sensitive than the other cholinesterases to inhibition by sarin, more sensitive to inhibition by TEPP, DFP, neostigmine and parathion, and as sensitive as the other enzymes to inhibition by tabun.

Sarin resembled tabun and DFP in combining with cholinesterase almost irreversibly during the first hour of their reaction. In contrast, the combination of TEPP or parathion with cholinesterase is slightly reversible (6, 19), while the combination of neostigmine with the enzyme is entirely reversible (20).

### *C. Solubility of sarin in an aqueous and lipoid medium, and comparison with the other anticholinesterase compounds*

Aqueous solutions of each anticholinesterase compound (concentrations as in Table I) were homogenized with equal volumes of peanut oil, following which the latter was removed by centri-

TABLE II

*Oral administration of sarin to ten normal subjects*

| Subject | Initial dose of sarin | | % Reduction of ChE* after initial dose | | Total amt. of sarin admn. over 3 days | | Maximum reduction of ChE* | |
|---|---|---|---|---|---|---|---|---|
| | *mg.* | *mg./Kg.* | Plasma | RBC | *mg.* | *mg./Kg.* | Plasma | RBC |
| 1. W. B. | 0.030 | 0.0005 | 10 | 10 | 0.426 | 0.007 | 14 | 33 |
| 2. E. M. | 0.031 | 0.005 | 10 | 10 | 0.428 | 0.007 | 29 | 27 |
| 3. S. M. | 0.123 | 0.002 | 10 | 15 | 2.70 | 0.044 | 69 | 93 |
| 4. C. L. | 0.137 | 0.002 | 18 | 20 | 3.00 | 0.044 | 81 | 93 |
| 5. W. T. | 0.472 | 0.008 | 37 | 38 | 5.66 | 0.096 | 86 | 97 |
| 6. O. L. | 0.560 | 0.008 | 32 | 46 | 6.72 | 0.096 | 81 | 98 |
| 7. W. R. | 0.956 | 0.016 | 60 | 72 | 3.82 | 0.064 | 87 | 98 |
| 8. T. C. | 1.309 | 0.016 | 57 | 68 | 7.85 | 0.096 | 88 | 99 |
| 9. W. M. | 1.540 | 0.022 | 69 | 85 | 7.14 | 0.102 | 88 | 99 |
| 10. C. C. | 1.760 | 0.022 | 69 | 72 | 8.36 | 0.102 | 84 | 99 |

* ChE activity is expressed as per cent of initial activity.

fugation and the anticholinesterase activity of the remaining aqueous solutions determined against plasma, red blood cell, brain and muscle cholinesterases (concentrations as in Table I). Sarin was more soluble in peanut oil than in water, 75 per cent being extracted from the aqueous solution. In this regard, sarin resembled DFP and parathion, of which 87 and 95 per cent were extracted, and was in contrast to TEPP and neostigmine, of which 6 and 16 per cent were extracted. Tabun was equally soluble in the two media, since 53 per cent was extracted.

*II. Effect of oral administration of sarin*

Sarin was administered orally in aqueous solution to eight normal subjects and in dilute propylene glycol to two others, over a 3 day period at intervals of 3 to 24 (average, 7.4) hours (Table II). Each dose was administered in 50 to 100 ml. tap water. At intervals before, during and after the period of administration of sarin, placebos

TABLE III

*Doses of orally administered sarin that produced signs and symptoms*

| Severity of signs and symptoms | Single dose (*mg./Kg.*) | Total dose (mg./Kg.) administered in increments of 0.008 to 0.016 mg./Kg. at an average interval of 7.4 hrs. over: | | |
|---|---|---|---|---|
| | | 24 hrs. | 48 hrs. | 72 hrs. |
| Mild | 0.022 | 0.032 | 0.060 | 0.088 |
| Moderate | 0.028 | 0.046 | 0.070 | 0.102 |

TABLE IV

*Signs and symptoms following oral administration of sarin*

| Subject | Muscarine-like | Nicotine-like | Central nervous system |
|---|---|---|---|
| 1. W. B. | 0 | 0 | 0 |
| 2. E. M. | 0 | 0 | 0 |
| 3. S. M. | 0 | 0 | Excessive dreaming, talking in sleep |
| 4. C. L. | Anorexia, mild abdominal cramps, excessive sweating | 0 | 0 |
| 5. W. T. | Anorexia, nausea, abdominal cramps, sweating, tightness in throat and chest, epigastric and substernal tightness with "heartburn" and eructation | Easy fatigue | Giddiness, tension, anxiety, excessive dreaming with nightmares |
| 6. O. L. | Anorexia, nausea, abdominal cramps, tightness in chest, epigastric and substernal tightness, bradycardia | Easy fatigue | 0 |
| 7. W. R. | Same as W. T., but tightness in chest more marked, with audible wheezing, prolonged expiration and subjective dyspnea | Easy fatigue | Headache, anxiety, tremor |
| 8. T. C. | Anorexia, tightness in chest, epigastric and substernal tightness, loose stools, excessive sweating, urinary frequency, subjective dyspnea with prolongation of expiratory phase | Easy fatigue, weakness, muscle twitching | Giddiness, insomnia, with excessive dreaming, tension, anxiety, jitteriness, headache, withdrawal and moderate depression, restlessness and emotional lability, tremor |
| 9. W. M. | Anorexia, nausea, abdominal cramps, tightness and mild pain in chest with wheezing, prolonged expiration and subjective dyspnea, epigastric and substernal tightness and pain, loose stools | Easy fatigue, weakness | Insomnia, tension, jitteriness, headache, giddiness |
| 10. C. C. | Anorexia, nausea, vomiting, abdominal cramps, tightness and discomfort in chest with wheezing respiration, prolonged expiration and some dyspnea, epigastric and substernal tightness and discomfort, eructation, sweating, salivation, lacrimation, tenesmus and urgent loose stools | Easy fatigue, weakness, muscle twitching and fasciculations, pallor | Insomnia, excessive dreaming, withdrawal, depression |

TABLE V

*Signs and symptoms produced by sarin*

| Site of action | Local effects: After conjunctival exposure | Local effects: Additional effects of exposure to vapor (13) |
|---|---|---|
| 1. Muscarine-like | | |
| Pupils | Miosis, marked or maximal, occasionally unequal | |
| Ciliary body | Frontal headache, eye pain on focussing, slight dimness of vision, occasional nausea and vomiting | |
| Conjunctivas | Hyperemia | |
| Nasal mucous membranes | | Rhinorrhea, hyperemia |
| Bronchial tree | | Tightness in chest, occasionally with prolonged wheezing expiration suggestive of bronchoconstriction or increased secretion |
| | **Systemic effects** | |
| | After oral administration | Additional effects of absorption by any route (13) |
| Gastrointestinal | Anorexia, nausea, vomiting, abdominal cramps, epigastric and substernal tightness (? cardiospasm) with "heartburn" and eructation, diarrhea, tenesmus | Involuntary defecation |
| Sweat glands | Increased sweating | |
| Salivary glands | Increased salivation | |
| Lacrimal glands | Increased lacrimation | |
| Heart | | Slight bradycardia |
| Bronchial tree | Tightness in chest, with prolonged wheezing expiration suggestive of bronchoconstriction or increased secretion, dyspnea, slight pain in chest | Increased bronchial secretion, pulmonary edema, with cyanosis |
| Pupils | | Miosis (variable) |
| Ciliary body | | Blurring of vision |
| Bladder | Frequency | Involuntary micturition |
| 2. Nicotine-like | | |
| Striated muscle | Easy fatigue, mild weakness, muscular twitching, fasciculations, generalized weakness | Cramps, weakness of muscles of respiration, with dyspnea |
| Sympathetic ganglia | Pallor, occasional elevation of blood pressure | |
| 3. Central nervous system | Giddiness, excessive dreaming, insomnia, nightmares, tension, anxiety, jitteriness, restlessness, emotional lability, headache, tremor, withdrawal and depression, reduction of voltage of EEG, bursts of slow waves of elevated voltage in EEG, especially on overventilation | Drowsiness, difficulty concentrating, slowness of recall, confusion, paresthesias, slurred speech, ataxia, generalized weakness, coma, with absence of reflexes, Cheyne-Stokes respirations, convulsions, depression of respiratory and circulatory centers |

of water and of dilute propylene glycol were administered.

### *A. General systemic effects*

*Taste.* Aqueous solutions of sarin had no taste, while solutions in dilute propylene glycol had the taste of the solvent. Except for this difference, the effects of the two solutions were the same. The highest concentration of sarin administered was 0.035 mg. per ml., in a 50 ml. amount.

*Dose that produced symptoms.* A single dose of 0.022 mg. per Kg. produced mild symptoms, and an additional 0.008 mg. per Kg. administered within eight hours resulted in moderate symptoms. The total doses required to produce symptoms when sarin was administered over 24 to 72 hour periods

are recorded in Table III. There was a narrow margin between the doses which produced mild symptoms and those which produced moderately severe symptoms.

*Time course of symptoms and occurrence of cumulative effects.* Symptoms began 20 to 60 minutes (average, 40 minutes) after the oral administration of sarin. Mild symptoms lasted for 10 minutes to 6 hours (average, 1 hour). Moderately severe symptoms lasted 5 to 24 hours. Following the disappearance of symptoms, there was increased susceptibility to the effects of sarin administered within 24 hours after the last dose. During this period, the upper limit of which was not determined, symptoms could be elicited by 0.008 to 0.016 mg. per Kg., doses which produced no symptoms when administered for the first time. The size of the dose necessary to produce a recurrence of symptoms, and the severity of these symptoms, varied inversely with the interval between doses. Thus, 0.008 mg. per Kg., administered at intervals of eight hours, produced symptoms after the fourth dose. These symptoms disappeared, but recurred with slightly greater severity after each subsequent dose. When the interval was greater, a larger dose was necessary to produce a recurrence of symptoms. Small divided doses of sarin totalling 0.03 mg. per Kg. per day which were insufficient to produce symptoms on the first day resulted in mild symptoms on the second and third days of administration, while larger divided doses totalling 0.04 mg. per Kg. per day produced more striking evidence of cumulation, as manifested by mild symptoms on the first day and severe symptoms on the third.

*Muscarine-like signs and symptoms.* The earliest symptoms to appear were usually anorexia, nausea, epigastric and substernal "tightness" (probably due to cardiospasm) with "heartburn" and eructation, and "tightness" in the chest (Tables IV and V). With larger doses of sarin, or repeated administration of small doses, there appeared abdominal cramps, vomiting, diarrhea, tenesmus, increased sweating and decreased skin resistance, increased salivation and lacrimation, slight bradycardia, pallor, urinary frequency, mild dyspnea with occasional wheezing, prolongation of expiration, and pain referred to the lower thoracic cage. Pupil size and blood pressure were not significantly changed (Table VI), except for an increase in systolic pressure of 35 mm. Hg in the subject with most marked symptoms. When respiratory symptoms were present, spirometric recording usually demonstrated a slight to moderate reduction in vital capacity and in the rate of movement of air into and out of the lungs, attributable to either bronchoconstriction or increased bronchial secretion. There was definite variation in the susceptibility of different subjects to respiratory symptoms. The subject in whom respiratory manifestations were most pronounced (W. R.) had a history of frequent upper respiratory tract infections which were at times associated with symptoms suggestive of mild asthma.

*Central nervous system signs and symptoms.* Following the earliest muscarine-like symptoms

TABLE VI

*Effect of oral administration of sarin on cardiac rate, pupil size, blood pressure, and skin resistance, and on response to atropine* *

| | Before admin. of sarin | During symptoms due to sarin | 12 hours after last dose of sarin |
|---|---|---|---|
| Cardiac rate (per minute) | 81 | 69 | 75 |
| Pupil size (mm.) | 6.1 | 4.8 | 5.5 |
| Blood pressure (mg. Hg) | 124/82 | 131/75 | 122/80 |
| Skin resistance Palms (ohms) | 230,000 | 86,000 | 260,000 |
| Dorsum of hands | 1,350,000 | 900,000 | 1,500,000 |
| Effect of atropine sulfate (1 mg. I.V.) on: | | | |
| Cardiac rate (per minute) | +30 | +18 | +25 |
| Pupil size (mm.) | +1.5 | +0.5 | +1.2 |
| Dryness of mouth | ++ | ± | + |
| Skin resistance Palms (ohms) | +860,000 | +300,000 | +650,000 |
| Dorsum of hands | +6,000,000 | +2,000,000 | +3,500,000 |

* Average values obtained under standard conditions in the eight subjects who had symptoms following sarin.

there appeared tension, anxiety, jitteriness, restlessness, emotional lability, giddiness, and insomnia with excessive dreaming and occasionally nightmares. With larger doses of sarin, or repeated administration of small doses, there appeared headache, tremor, withdrawal, and mild or moderate depression. Following small doses of sarin which did not cause symptoms there was no change in the electroencephalogram. With the appearance of the mildest symptoms there was noted a slight, but definite diminution in the voltage of the electroencephalogram, which in some subjects was more noticeable in the left than in the right hemisphere, especially in the occipital leads. When moderate symptoms were present, even though intermittently, there appeared in the electroencephalogram more irregularities in rhythm, variation in potential, and intermittent bursts of abnormal waves similar to those seen in patients with epilepsy (12, 13). These consisted of slow waves (two and one-half to six per second) of elevated voltage (70 to 150 mV.), usually most marked in the frontal leads. They were usually not striking prior to hyperventilation. In general, more striking electroencephalographic changes occurred in those subjects who showed greater lability of pattern (though within the limits of normal) in their control records than in those who had very regular patterns of low voltage. Following the cessation of sarin administration, the electroencephalographic changes diminished, but could be detected for 4 to 18 days after the disappearance of symptoms.

*Nicotine-like signs and symptoms.* All the subjects who had muscarine-like and central nervous system symptoms also had some increased fatigability, and three had mild generalized weakness, especially after exertion. Two had some involuntary muscular twitching and fasciculations. One developed moderate pallor.

*Effect of atropine on symptoms due to sarin.* The intravenous administration of atropine sulfate (1 mg.) was invariably followed by prompt relief of all the muscarine-like symptoms due to sarin and by an increased feeling of well-being. There was less striking improvement in the central nervous system symptoms, and usually a decrease in the voltage of the electroencephalogram (more than after atropine alone), a decrease in the irregularities of frequency and potential, and a decrease in the frequency of appearance of slow waves of elevated voltage. The slow waves did not entirely disappear; they would occur after a longer period of hyperventilation than was required to bring them out prior to the administration of atropine.

*Effect of sarin on the action of atropine.* In the presence of moderately severe symptoms due to sarin, there was reduced susceptibility to the action of atropine, as indicated by the effect of intravenous administration of 1 mg. of this drug on cardiac rate, pupil size, dryness of mouth, and skin resistance (Table VI). Following the disappearance of symptoms due to sarin, the response to atropine returned, and was only slightly less than prior to sarin administration.

*Effect of inhaled tobacco smoke.* The casual smoking of one or two cigarettes was usually promptly followed by an increase in the gastrointestinal and respiratory symptoms. In one subject, spirometric recording revealed a further decrease in the vital capacity and in the rate of movement of air into and out of the lungs.

### *B. Effect on plasma and red blood cell cholinesterase activity*

Prior to sarin administration the cholinesterase activity of each subject was in the normal range: for plasma the range was 0.85 to 1.26 ΔpH per hour, and the mean, standard deviation, and standard error were 1.02, 0.15, and 0.05 ΔpH per hour, respectively; for red blood cells the range was 0.55 to 0.96 ΔpH per hour, and the mean, S.D.,

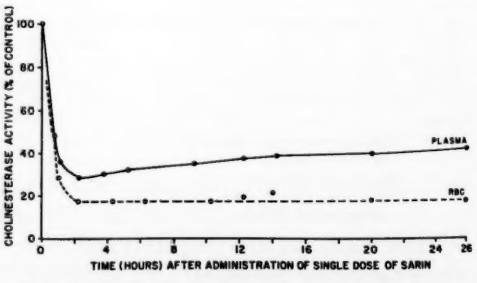



FIG. 2. DEPRESSION OF PLASMA AND RED BLOOD CELL CHOLINESTERASE ACTIVITY AFTER A SINGLE ORAL DOSE OF SARIN, AND RESTORATION DURING THE FIRST DAY

Average values obtained in eight subjects. The average dose of sarin was 0.012 mg. per Kg.

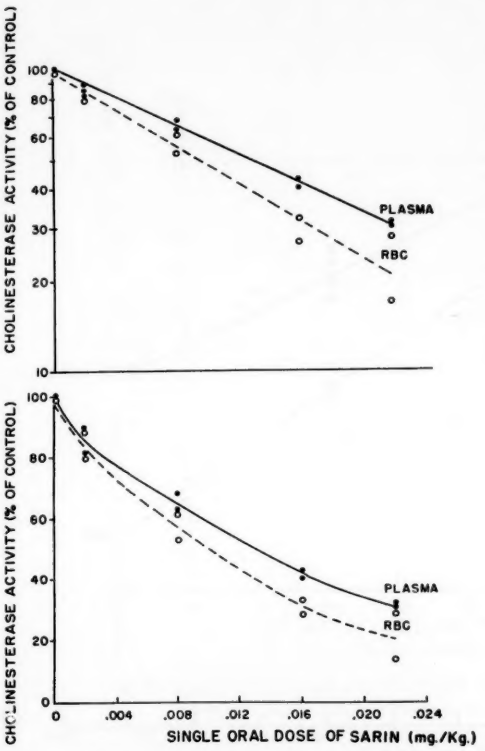



FIG. 3. RELATION BETWEEN THE SIZE OF A SINGLE ORAL DOSE OF SARIN AND THE RESULTING DEPRESSION OF PLASMA AND RED BLOOD CELL CHOLINESTERASE ACTIVITY

The same data are presented in the upper graph on a logarithmic scale, and in the lower graph on a linear scale.

and S.E., 0.72, 0.13, and 0.04 ΔpH per hour, respectively.

*Effect of a single dose.* The oral administration of from 0.002 to 0.022 mg. per Kg. of sarin resulted in from 15 to 75 per cent reduction in plasma and red blood cell cholinesterase activity. Maximum depression occurred in one to two hours (Figure 2). The degree of depression of the cholinesterase activity of the red blood cells was slightly greater than that of the plasma, paralleling the relative sensitivity of these enzymes to sarin *in vitro*. The dose-effect relationship (Figure 3) was similar to that found when varying concentrations of sarin were added to cholinesterase preparations *in vitro* (Figure 1). Each increment in dose inhibited the same *fraction* of cholinesterase, and had a diminishing effect on the *amount* inhibited. The logarithm of the fraction of cholinesterase that remained active two hours after the administration of sarin ($ChE_1/ChE_0$) decreased linearly with increasing dose of sarin (S), or, the logarithm of the reciprocal of the fraction that remained active was directly proportional to the dose.

*Effect of repeated doses.* Repeated doses administered orally at intervals of 3 to 24 (average, 7.4) hours over a period of 3 to 3.5 days, in total dose of up to 0.102 mg. per Kg., resulted in progressive diminution in the cholinesterase activity of the plasma to near 15 per cent of original activity (Table II and Figure 4). The intervals between doses of sarin were usually long enough for some restoration of plasma cholinesterase activity to take place (Figure 4), while that of the red blood cells was not appreciably restored during these intervals. This is believed to account for the progressively greater depression of red blood cell than of plasma cholinesterase activity.

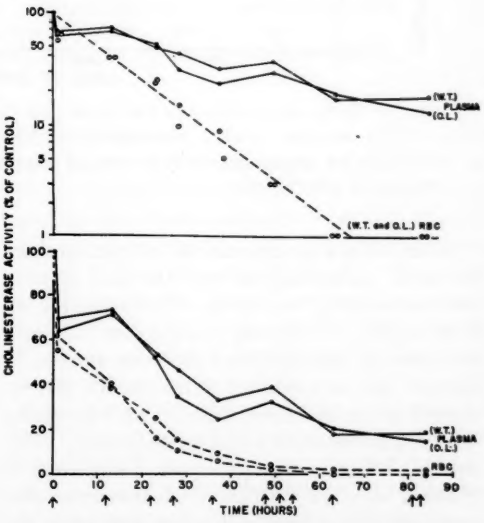



FIG. 4. EFFECT OF THE REPEATED ORAL ADMINISTRATION OF SARIN ON PLASMA AND RED BLOOD CELL CHOLINESTERASE ACTIVITY OF TWO SUBJECTS

The upper graph represents the same data plotted on a logarithmic scale. Each arrow represents the oral administration of 0.008 mg. per Kg. of sarin.

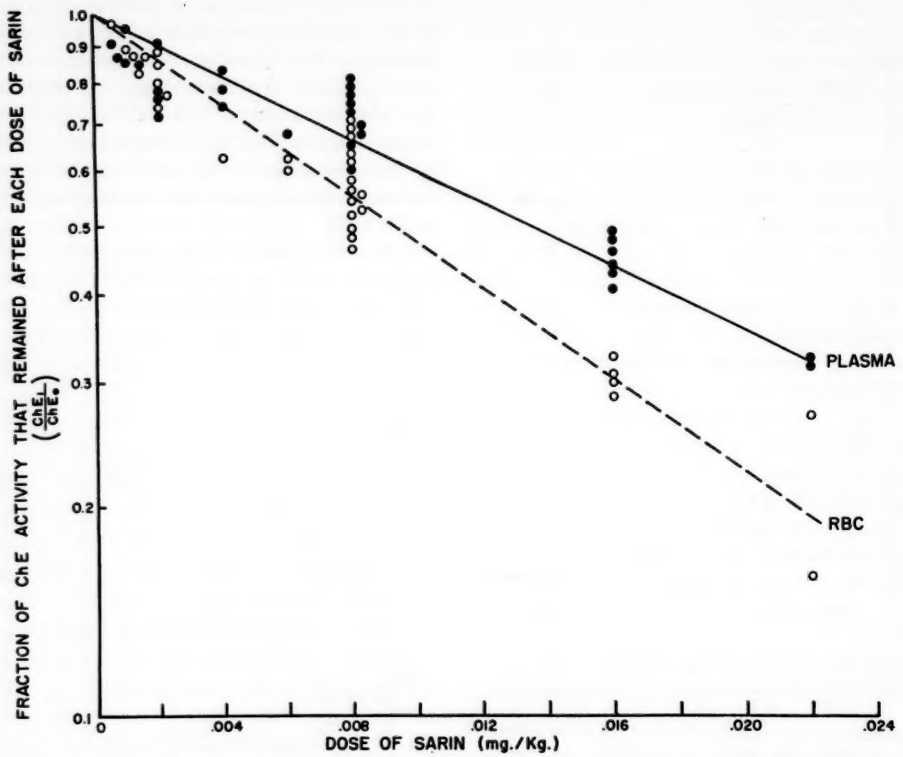



FIG. 5. EFFECT ON PLASMA AND RED BLOOD CELL CHOLINESTERASE ACTIVITY OF REPEATED DOSES OF SARIN ADMINISTERED AT VARIOUS LEVELS OF ACTIVITY

$ChE_0$ is the enzyme activity at the time of administration of any dose of sarin, and $ChE_1$ the activity two hours after administration.

When sarin was administered in repeated doses the same relationship between the dose of sarin and the resulting depression of cholinesterase activity applied as following a single dose; *i.e.*, each increment in dose inhibited the same fraction of enzyme, and the logarithm of the fraction that remained active decreased linearly with increasing dose, regardless of the initial level of activity (Figure 5). When the repeated doses of sarin were of constant size, the fraction of cholinesterase that remained active after each dose, and hence the fraction inhibited by each dose, were constant and were independent of the level of activity at the time each dose was administered (Figure 6):

$$\frac{ChE_1}{ChE_0} = k, \quad \text{and} \quad \frac{ChE_0 - ChE_1}{ChE_0} = 1-k,$$

where $ChE_0$ equals cholinesterase activity just before the administration of each dose of sarin, and $ChE_1$ equals cholinesterase activity two hours after each dose. In contrast, the *amount* of cholinesterase inhibited by each successive dose of sarin ($ChE_0 - ChE_1$) decreased progressively, as it was proportional to the activity at the time the dose was administered ($ChE_0$) (Figure 4).

Since the restoration of red blood cell cholinesterase activity during the interval between doses of sarin was very slight, the effect of repeated doses of sarin on this enzyme was almost entirely cumulative until the enzyme was depressed below approximately three per cent of the original activity. The logarithm of the fraction of red blood cell cholinesterase that remained active at any time

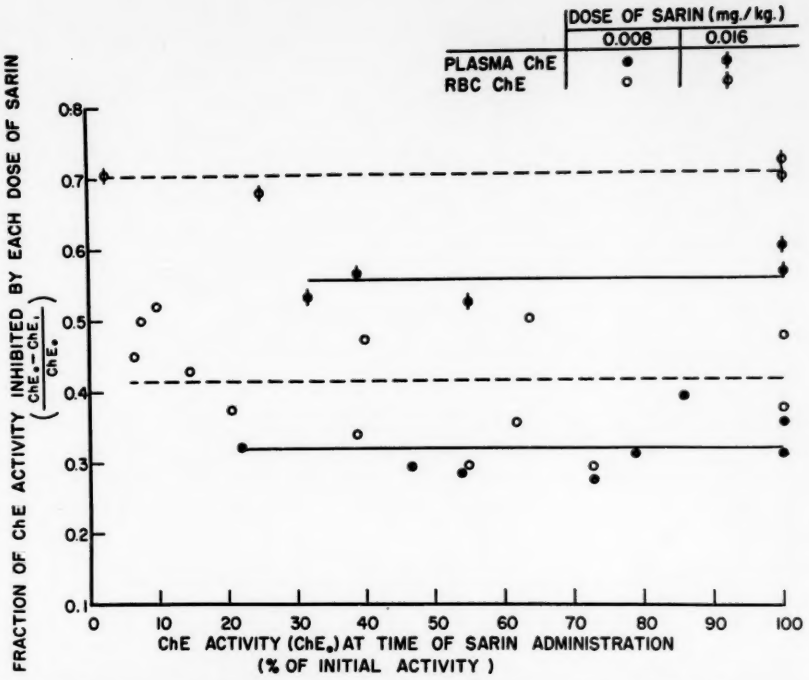



FIG. 6. EFFECT ON PLASMA AND RED BLOOD CELL CHOLINESTERASE ACTIVITY OF TWO DIFFERENT DOSES OF SARIN ADMINISTERED REPEATEDLY AT VARIOUS LEVELS OF ACTIVITY

$ChE_0$ is the enzyme activity at the time of administration of any dose of sarin, and $ChE_1$ the activity two hours after administration.

during the three day period of administration decreased linearly with the total amount of sarin that had been administered up to that time, until the activity of the enzyme was reduced below three per cent of the original value (Figure 7). At this low level of activity the slow restoration of enzyme activity between doses of sarin became significant, as did technical errors in the determination. During the three day period of administration the effect of repeated doses of sarin on red blood cell cholinesterase (Figure 7) was approximately the same as the effect of equal single doses (Figure 3 and its extrapolation in Figure 8), until the activity was depressed to the point where the daily rate of restoration became significant.

### *C. Rate of restoration of plasma and red blood cell cholinesterase activity*

Following the oral administration of sarin, maximum depression of plasma and red blood cell cholinesterase activity occurred within one to two hours. After depression to near zero activity, plasma cholinesterase was restored at an average rate of approximately 4 per cent of original activity during the first 3 hours, 7 per cent during the first 24 hours (Figure 2). The rate of restoration of the plasma enzyme was maximal during the first 24 hours, and was approximately 8 per cent of original activity on the second day, 6 per cent on the third, 5 per cent on the fourth, 4 per cent on the fifth and sixth, 3 per cent per day through the ninth, 2 per cent per day through the sixteenth day, and 1 per cent per day after that time until the activity returned to its original level at the end of about 40 days (Figure 9).

Following depression of red blood cell cholinesterase to near zero, the activity of this enzyme was restored at a rate of approximately 1.1 per cent of original activity per day, over a period of 3 months. This rate was constant, with the pos-

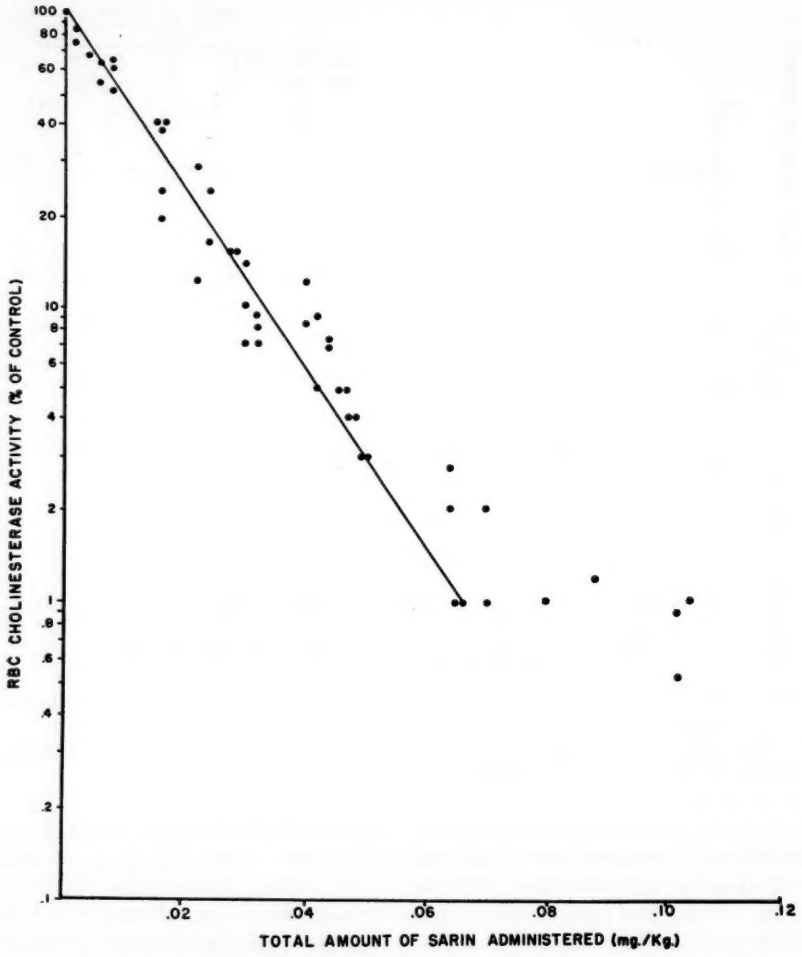



FIG. 7. EFFECT OF THE REPEATED ORAL ADMINISTRATION OF SARIN ON RED BLOOD CELL CHOLINESTERASE ACTIVITY

The cholinesterase activity and total dose of sarin are recorded after each of the several doses that were administered to 10 subjects.

sible exception of the first day, when the increase in red blood cell enzyme activity may have been slightly greater (1.7 per cent of original activity).

Following less marked depression of plasma and red blood cell cholinesterases, the daily rates of restoration of enzyme activity were less, but the times required for complete restoration (40 and 92 days) were the same.

The rate of restoration of plasma cholinesterase activity was increased transiently by the intravenous administration of plasma or whole blood, and red blood cell cholinesterase activity by whole blood, but this neither ameliorated nor prevented symptoms due to sarin.

When plasma and red blood cells were withdrawn from subjects who had received sarin and allowed to stand or dialyze at 0, 5 or 24° C. for several weeks, no restoration of cholinesterase activity could be demonstrated, indicating irreversible inactivation of these enzymes.

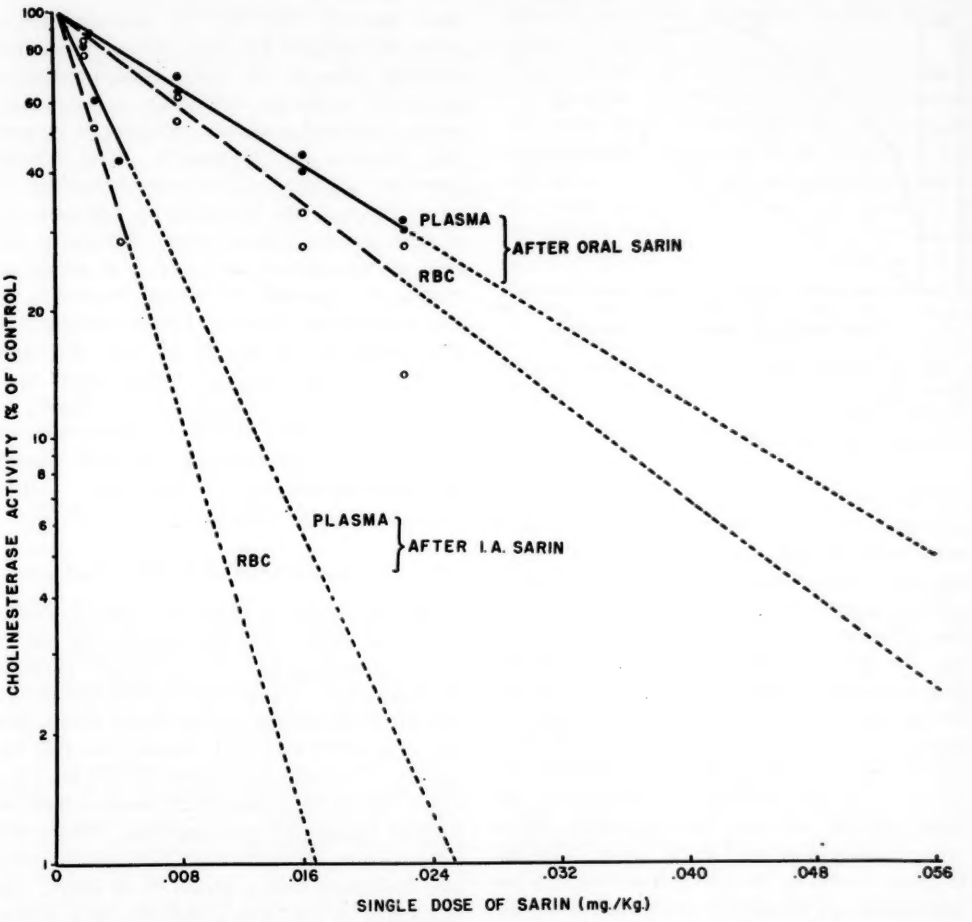



FIG. 8. COMPARISON BETWEEN THE EFFECTS OF SINGLE DOSES OF ORALLY AND INTRA-ARTERIALLY ADMINISTERED SARIN ON PLASMA AND RED BLOOD CELL CHOLINESTERASE ACTIVITY

The dotted lines represent extrapolation from data.

*D. Relation of symptoms to plasma and red blood cell cholinesterase activity*

Following a single oral dose of sarin, symptoms usually began coincident with the depression of plasma and red blood cell cholinesterase activity to approximately 34 and 22 per cent of original activity. Following repeated doses there was no correlation between the onset of symptoms and the precise level of activity of the plasma and red blood cell enzymes, except that this was depressed below these values. The cholinesterase activity of the red blood cells could be gradually depressed to near zero by repeated doses administered over a period of several days without symptoms necessarily ensuing, or without any relation to the symptoms that occurred. Symptoms usually disappeared before any restoration of red blood cell cholinesterase occurred, and after little or no restoration of the plasma enzyme.

*E. Absence of other effects of sarin than those attributable to the inhibition of cholinesterase enzymes*

There was no change in the hematocrit, hemoglobin, red cell count, white blood count, differ-

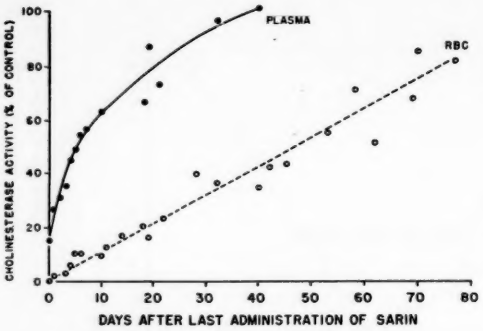



FIG. 9. RATE OF RESTORATION OF PLASMA AND RED BLOOD CELL CHOLINESTERASE ACTIVITY AFTER CESSATION OF SARIN ADMINISTRATION

Average values obtained in 10 subjects.

ential formula, number of circulating eosinophils, sedimentation rate, clot retraction time, blood nonprotein nitrogen, fasting blood sugar, or serum chloride, carbon dioxide combining power, calcium, phosphorus, cholesterol, uric acid, alkaline phosphatase, bilirubin, albumin and globulin. The blood coagulation time increased slightly, from a mean of 7 minutes to 13 minutes. The following tests of hepatic and renal function remained normal: thymol turbidity, cephalin flocculation, phenolsulphonphthalein excretion and urinalysis. The electrocardiogram, chest X-ray, and basal metabolic rate were also unchanged, and no occult blood was detected in the stools. The lack of effect of sarin on the fasting blood sugar, white blood count and number of circulating eosinophils, and the slight effect on blood pressure, indicate that the amounts of sarin that were administered did not cause any appreciable adrenalin release as a result of stimulation of the preganglionic autonomic nerve endings in the adrenal medulla.

*III. Effect of intra-arterial administration of sarin*

The injection of 0.003 and 0.005 mg. per Kg. of sarin into the brachial artery of two subjects produced local effects in the injected extremity and systemic effects. The local effects included profuse sweating (despite the prior intramuscular administration of 2.25 mg. atropine sulfate), reduction of grip strength by about 50 per cent, and fasciculations which appeared after exercise. The systemic effects were similar to those that were observed after the oral administration of sarin and included anorexia, nausea, generalized sweating (despite the prior administration of atropine), giddiness (especially on standing), some generalized weakness, twitching of leg muscles, insomnia and nightmares. The sweating lasted for 12 hours. Electroencephalograms obtained two and four days after the administration of sarin revealed a slight overall decrease in voltage and intermittent bursts of slow waves, occurring at a frequency of one and one-half to two and one-half per second. Plasma cholinesterase was reduced to 61 and 42 per cent of original activity, and red blood cell cholinesterase to 52 and 28 per cent of original activity (Figure 8). These changes were similar to those which followed the oral administration of approximately three and one-half times as much sarin. This ratio is the same as for DFP (1–4) and TEPP (6).

*IV. Effect of conjunctival instillation of sarin*

The instillation of sarin (in aqueous solution or normal saline) into the conjunctival sac resulted in striking and very prolonged miosis of the treated eye. The lowest concentration of sarin that produced definite and persistent miosis when one drop (0.03 ml.) was instilled was 0.01 mg. per ml. This amount of sarin (0.0003 mg.) produced within 10 minutes local miosis which was marked, though not quite maximal. There were no subjective manifestations. The miosis gradually diminished over a period of 60 hours. The instillation of one drop (0.03 ml.) of a solution containing 0.03 mg. per ml. of sarin resulted within 10 minutes in miosis which was nearly maximal. This extreme miosis persisted for several minutes and was followed by submaximal pupillary constriction which remained severe for 72 hours, and disappeared after about 90 hours. There were mild local symptoms of "pressure" in the treated eye and the sensation that the eye "felt smaller and had sunk back into the head." These local effects of sarin were not associated with any change in plasma or red blood cell cholinesterase activity. They were not appreciably influenced by the intravenous administration of 1 mg. atropine sulfate, but were ameliorated by the repeated conjunctival instillation of one per cent atropine or two per cent homatropine.

## DISCUSSION

The effects observed after administration of sarin, and those which have occurred after more marked exposure than in this study (13) (Table V), are similar to the effects of other organic phosphate anticholinesterase compounds, such as DFP (1), TEPP (6) and parathion (8). They are entirely attributable to the inhibition of cholinesterase enzymes in the effector tissues and resultant local accumulation of excessive concentrations of acetylcholine wherever this compound is normally liberated: at the endings of the parasympathetic nerves to the smooth muscle of the iris, ciliary body, bronchial tree, gastrointestinal tract, bladder, and blood vessels, to the secretory glands of the respiratory tract and to cardiac muscle, and of sympathetic nerves to the sweat glands (resulting in "muscarine-like" signs and symptoms); at the endings of motor nerves to voluntary muscle and in the autonomic ganglia (resulting in "nicotine-like" signs and symptoms); and in the central nervous system. The effects of sarin, like those of the other organic phosphate anticholinesterase compounds, can be classified as local or systemic. Local effects on the smooth muscle of the eye and on the conjunctival blood vessels occurred after local ocular exposure, and local effects on the smooth muscle and secretory glands of the respiratory tract occur after inhalation of sarin vapor (13). Systemic effects follow sufficient absorption by any route. Since gastrointestinal symptoms were more pronounced after ingestion of sarin than after comparable absorption by other routes (13), it is possible that these may be the result of local as well as systemic action.

The effects of systemic absorption of sarin on the eye were negligible, in the doses administered, and even more marked systemic exposure has minimal effect on the eye in the absence of local exposure (13). The effect of systemic absorption on the smooth muscle and secretory glands of the respiratory tract, as indicated by symptoms and signs compatible with bronchoconstriction or increased bronchial secretion, were slight except for one subject who had a history suggestive of mild asthma. More marked local or systemic exposure may result in a considerable increase in bronchial secretion, but bronchoconstriction has not been prominent in man (13), in contrast to most experimental animals (15, 16). Systemic absorption of sarin did not produce evidence of vasodilatation, in contrast to the local effect on conjunctival vessels; in fact, a slight rise in blood pressure occurred in one subject, and is common after more marked exposure (13), presumably owing to stimulation of sympathetic ganglia or release of adrenalin. The effect on heart rate was slight. The effect on the gastrointestinal tract, sweat glands, central nervous system, and skeletal muscle was more pronounced.

Of the compounds studied, sarin had the greatest anticholinesterase activity *in vitro;* it is the most toxic to animals; and it produced its pharmacologic effects in man in the smallest doses. TEPP, DFP and parathion were less potent, in this order. There was a rough correlation between the inhibitory activity of these compounds

TABLE VII

*Comparison between the anticholinesterase activity in vitro of sarin and other organic phosphate anticholinesterase compounds, and the effects in rats and man of the intramuscular (I.M.), intra-arterial (I.A.) and oral administration of these compounds* *

| | *In vitro* | In rats | | In man | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ratio of antiChE activity | $LD_{50}$ for rats (*mg./Kg.*) | | Dose (*mg./Kg.*) that produced 50% depression of RBC ChE | | Dose (*mg./Kg.*) that produced moderate symptoms | | Estimated lethal dose (*mg./Kg.*) | |
| | | I.M. | Oral | I.M. or I.A. | Oral | I.M. or I.A. | Oral | I.M. | Oral |
| Sarin | 1 | 0.17 | 0.6 | 0.003 (I.A.) | 0.01 | 0.006 (I.A.) | 0.028 | 0.03 | 0.14 |
| Tabun | 1/5 | 0.80 | 3.7 | | | | | | |
| TEPP | 1/10 | 0.65 | 1.4 | 0.025 (I.M.) | 0.10 | 0.083 (I.M.) | 0.35 | 0.38 | 1.7 |
| DFP | 1/100 | 1.8 | 6 | 0.07 (I.M.) | 0.28 | 0.083 (I.A.) | 0.32 ? | 0.48 | 2.1 |
| Parathion | 1/4,000 | 6 | 10 | | | | | | |

* See the following references: $LD_{50}$ for rats of sarin (21), tabun (22), TEPP (23), DFP (24) and parathion (25). Doses that produced symptoms in man: TEPP (8) and DFP (1).

TABLE VIII

*Estimation of the amount of sarin required to produce 50 per cent inhibition of the blood and tissue cholinesterases of a 70 Kg. man, and comparison with the oral and parenteral doses that produced 80 per cent inhibition of red blood cell cholinesterase in a 70 Kg. man*

| Body fluid or tissue | Total weight (*Kg.*) | Mg. sarin required to produce 50% inhibition of ChE activity of | |
|---|---|---|---|
| | | 1 mg. of body fluid or tissue *in vitro* | Total body fluid or tissue (calculated) |
| Plasma | 3.46 | $11.5 \times 10^{-9}$ | 0.0400 |
| Red blood cells | 2.84 | $12.3 \times 10^{-9}$ | 0.0349 |
| Muscle (skeletal) | 29.40 | $8.7 \times 10^{-9}$ | 0.2560 |
| Cerebrum | 1.15 | $3.7 \times 10^{-9}$ | 0.0042 |
| Cerebellum | 0.15 | $12.0 \times 10^{-9}$ | 0.0012 |
| Thalamus | 0.02 | $28.0 \times 10^{-9}$ | 0.0006 |
| Pons, medulla, and spinal cord | 0.04 | $5.1 \times 10^{-9}$ | 0.0002 |
| Liver | 1.40 | $3.2 \times 10^{-9}$ | 0.0045 |
| Spleen | 0.15 | $2.1 \times 10^{-9}$ | 0.0003 |
| Kidneys | 0.26 | $0.9 \times 10^{-9}$ | 0.0002 |
| Pancreas | 0.09 | $1.2 \times 10^{-9}$ | 0.0001 |
| Heart | 0.31 | $0.7 \times 10^{-9}$ | 0.0002 |
| | | Total | 0.3424 |
| | | Mg. sarin that produced 50% inhibition of red blood cell ChE activity and 40% inhibition of plasma ChE | |
| Man | 70 | After intra-arterial admin. | 0.20 |
| | | After oral administration | 0.70 |

against the "specific" cholinesterases of red blood cells, muscle and brain, and the effective dose in animals and man, but the differences in the former were greater (Table VII). It is likely that the pharmacologic effects and toxicity of these compounds depend not only on the degree of their anticholinesterase activity, but also on their accessibility to the cholinesterase enzymes of the various effector tissues and on the time course of inhibition of these enzymes. Accessibility appears to be influenced by relative solubility in aqueous and lipoid media. Sarin, DFP and parathion, which are more soluble in lipoid than aqueous medium, have much greater central neural action than TEPP and neostigmine, which are more soluble in aqueous medium. DFP and parathion, especially the latter, are more toxic than might be expected from their anticholinesterase activity *in vitro*. This may be due in part to their greater partition coefficients in lipoid medium. In addition, parathion is converted *in vivo* into the oxygen analogue, a much more potent inhibitor of cholinesterase than the parent compound, which owes its anticholinesterase activity even *in vitro* to the presence of small amounts of the oxygen analogue or of the thio-ethyl or thio-phenyl isomers (26).

The time course of the inhibition of cholinesterase enzymes by anticholinesterase compounds depends on the rate of inhibition, reversibility of the inhibition, rate of restoration of the enzymes by the tissues, and rate of removal of free anticholinesterase compound. Sarin combines rapidly with cholinesterase enzymes *in vitro*, and after a short period of time this combination is not spontaneously reversible *in vitro*. In this regard sarin resembles DFP (1), and differs from TEPP (6) and parathion (19), which combine with cholinesterase in a reversible manner for several hours before the combination becomes "irreversible." The rate of restoration of the plasma and red blood cell enzymes *in vivo* following depression by sarin was similar to that following DFP (1), and is believed to reflect the rate of regeneration of plasma enzyme by the liver and of red cell enzyme in newly formed red blood cells, which are replaced at the rate of approximately one per cent per day. The rate of removal of free anticholinesterase compound is determined by the rates of hydrolysis, combination with cholinesterase enzymes and other proteins, other means of detoxification, and excretion. Sarin hydrolyzes slowly in dilute aqueous solutions at 37° C. and pH 7 over a period

of approximately 24 hours. Hence this would be expected to have little influence on the action of sarin *in vivo,* which is exerted within an hour after administration. However, in the presence of tissue extracts, especially of liver, the hydrolysis of DFP is accelerated by the action of a hydrolytic enzyme (phosphofluorase) (*27*). It is not known if enzymatic hydrolysis of sarin occurs in man; the rates of disappearance, detoxification, and excretion of this compound have not been ascertained.

In order to estimate the proportion of orally or parenterally administered sarin which is hydrolyzed, detoxified or excreted before inactivating cholinesterase enzymes, the amount of sarin which might inhibit 50 per cent of the cholinesterase activity of the blood and other tissues of a 70 Kg. man was calculated (Table VIII). This figure was computed from the concentrations of sarin required to inhibit the enzyme activity of the various tissues *in vitro,* and from the estimated weight of these tissues. Since the sensitivities of red blood cell, muscle and brain cholinesterases to sarin *in vitro* were equal, and since oral or intramuscular administration to man of organic phosphate anticholinesterase compounds for *short* periods of time resulted in nearly as much depression of muscle and brain cholinesterases as of red blood cell enzyme (*28*), it has been assumed in the calculation that sarin is uniformly distributed in the blood and other tissues, even though it is more likely that the red blood cell enzyme is depressed to a somewhat greater degree than the cholinesterases of other tissues. The figure (0.34 mg.) thus obtained for the amount of sarin that might be expected to produce 50 per cent inhibition of the cholinesterase activity of the blood and other tissues of a 70 Kg. man approximates the dose (0.21 mg.) that produced 50 per cent inhibition of red blood cell enzyme activity after intra-arterial administration. This suggests that intravascularly injected sarin is not hydrolyzed or detoxified to an appreciably greater extent *in vivo* than *in vitro,* and that it is not excreted prior to its reaction with cholinesterase. The calculations also suggest that about 75 per cent of the sarin that reacted with cholinesterase may have combined with muscle enzyme, about 22 per cent with plasma and red blood cell enzymes, and about 3 per cent with those of brain and liver. Although skeletal muscle has less cholinesterase activity per unit of weight than plasma, red blood cells, and some parts of the brain, and reacts with less sarin per unit of weight, its larger mass (42 per cent of body weight) would react with more sarin than all other tissues combined.

The observation that the amount of cholinesterase inhibited by a given dose of sarin is proportional to the level of enzyme activity suggests that there is a time limit for the reaction between cholinesterase and sarin. While the rate of the reaction would be expected to depend on the concentration of enzyme, the extent of the reaction would not, unless the reaction were limited by time. Such limitation could occur as a result of competitive removal of sarin by combination with proteins other than cholinesterase, by hydrolysis of sarin, and by competition with acetylcholine for cholinesterase. The first is probably most important. It is likely that the dose-effect relationship observed for the action of sarin on plasma and red blood cell cholinesterase activity may also be applicable to the effect of sarin on tissue cholinesterases and on the function of cholinergic effector organs. Riker and Wescoe (*29*) have demonstrated that the effect of intra-arterial DFP on the cholinesterase activity and response to electrical stimulation of the cat submaxillary gland is proportional to the level of enzyme activity.

The signs and symptoms produced by sarin are due to inhibition of the cholinesterase enzymes of the nervous system, muscle and secretory glands, and not to the coincident inhibition of the enzymes of the plasma and red blood cells. However, the activity of the latter enzymes may be used as a guide of some value in detecting systemic absorption of sarin, and even in estimating the amount absorbed, over a period of up to several days. Local ocular and respiratory (13) effects of sarin may occur without any inhibition of plasma and red blood cell cholinesterase activity, but systemic manifestations are invariably accompanied by depression of the activity of these enzymes. Following a single oral dose of sarin symptoms usually began coincident with depression of the plasma and red blood cell enzymes to approximately 34 to 22 per cent of original activity, and, following a single intravascular dose, to 60 and 50 per cent of original activity. The depression of these enzymes accompanying onset of

symptoms was thus less marked following rapid than gradual absorption of sarin, probably because rapid absorption allows less "buffering" action of plasma and red blood cell cholinesterases and more ready access of the sarin to the enzymes of other tissues.

When the period of exposure to sarin was brief, as after a single dose, the degree of depression of plasma and red blood cell cholinesterases could be correlated roughly with the occurrence and severity of symptoms, and presumably with the degree of depression of the enzymes of the effector tissues. Observations following exposure to DFP (1–4), TEPP (6) and parathion (8) indicate that the organic phosphate anticholinesterase compounds exert their effects by depressing the cholinesterase activity of the tissues below a threshold at which changes in function begin, and that there is a relatively narrow margin between this threshold and the level of enzyme activity below which serious and even fatal functional alterations occur. The former threshold has been estimated to be about 50 per cent of normal activity, and the latter 10 to 20 per cent of normal (28).

When the period of exposure to sarin was longer, as after repeated doses at intervals of several hours or days, there was no correlation between the onset or severity of symptoms and the precise level of cholinesterase activity of the plasma and red blood cells, except that this was depressed considerably below normal. These observations, and the disappearance of symptoms before there was appreciable restoration of plasma and red blood cell enzyme, suggest that either the restoration of cholinesterase enzymes of the effector tissues following depression by sarin is more rapid than that of the plasma and red blood cells, or return of function is not dependent solely on recovery of tissue cholinesterase activity. The complete restoration of cholinesterases in the effector tissues probably requires several days, as indicated by increased susceptibility to the effects of sarin for more than 24 hours after the disappearance of symptoms, and persistence of electroencephalographic changes for as long as 18 days. Increased susceptibility to the effects of further exposure to sarin probably persists until the enzymes of the effector tissues have been restored. The cumulative effect of repeated exposure may result in the sudden appearance of severe symptoms, since the initial depression of activity may not be associated with warning symptoms.

It is likely that a single exposure of man to an organic phosphate anticholinesterase compound which was sufficient to reduce the enzyme of the red blood cells to between 1 and 5 per cent of original activity would lower the activity of other tissues below 10 per cent of normal, and would probably be lethal (13, 28). This "lethal" dose can be estimated by extrapolation of the observed dose-effect relationships for sarin (Figure 8), DFP (1) and TEPP (6) (Table VIII). Death due to these compounds, or to parathion, is caused by respiratory failure resulting from weakness of the muscles of respiration, central depression of respiration, and airway obstruction by bronchial and salivary secretions and to a lesser extent bronchoconstriction (8, 13). Treatment has relied upon the administration of large doses of atropine, alleviation of airway obstruction, and artificial respiration when needed. Atropine ameliorates the muscarine-like actions of the anticholinesterase compounds, and to a lesser extent the central neural effects, by inhibiting the action of acetylcholine at these sites. There is reduced susceptibility to the action of atropine in the presence of symptoms due to sarin, so that large doses may be administered before signs of atropinization appear (13). Atropine has no appreciable influence on muscular weakness. Until now there has been no practical means of accelerating recovery of cholinesterase activity and of muscular strength following depression by anticholinesterase compounds. Recent work, however, indicates that a number of oximes, including 2-pyridine aldoxime (2-PAM) and diacetyl monoxime (DAM), reverse cholinesterase inhibition (30) and neuromuscular block (31, 32) due to sarin, and accelerate the return of muscular strength.

## SUMMARY

The administration of sarin to normal subjects resulted in muscarine-like, nicotine-like, and central nervous system signs and symptoms attributable to the inhibition of cholinesterase enzymes in the effector tissues, and resembling those produced by other organic phosphate anticholinesterase compounds. Of the compounds studied, sarin had the greatest anticholinesterase activity *in vitro;* it

is the most toxic to animals; and it produced its pharmacologic effects in man in the smallest doses. TEPP, DFP and parathion were less potent, in this order. Sarin resembled DFP and parathion in being more soluble in lipoid than aqueous medium, and in producing marked central neural effects. It resembled DFP in producing "irreversible" inhibition of cholinesterase enzymes *in vitro,* and probably of plasma and red blood cell cholinesterase activity *in vivo.*

The relationship between the dose of sarin and the degree of depression of cholinesterase activity was the same *in vitro* as it was for plasma and red blood cell cholinesterase activity *in vivo:* the amount of enzyme inhibited by a given dose was proportional to the level of enzyme activity; each increment in dose inhibited the same fraction of enzyme; and the logarithm of the fraction that remained active decreased linearly with increasing dose. When sarin was administered in repeated doses at intervals of several hours to one day, the effect on cholinesterase activity and on symptoms was cumulative.

The oral administration of sarin resulted in systemic effects, and perhaps local gastrointestinal actions. Intra-arterial injection produced local and systemic effects. Conjunctival instillation resulted in local ocular changes. The effects of sarin were very prolonged, lasting from several hours after the smallest effective doses to several days after doses which produced moderate symptoms. The administration of atropine ameliorated the muscarine-like effects of sarin, and to a lesser extent the central neural effects, but had no influence on muscular weakness or fasciculations. There was reduced susceptibility to the action of atropine in the presence of symptoms due to sarin.

Following depression by sarin, the cholinesterase activity of the plasma was restored over a period of 40 days, at a rate compatible with the regeneration of enzyme by the liver, while that of the red blood cells was restored at the rate of one per cent per day, suggestive of regeneration of enzyme in newly formed red blood cells.

## REFERENCES


1. Grob, D., Lilienthal, J. L., Jr., Harvey, A. M., and Jones, B. F. The administration of di-isoprophyl fluorophosphate (DFP) to man. I. Effect on plasma and erythrocyte cholinesterase; general systemic effects; use in study of hepatic function and erythropoiesis; and some properties of plasma cholinesterase. Johns Hopk. Hosp. Bull. 1947, **81**, 217.
2. Grob, D., Lilienthal, J. L., Jr., and Harvey, A. M. The administration of di-isopropyl fluorophosphate (DFP) to man. II. Effect on intestinal motility and use in the treatment of abdominal distention. Johns Hopk. Hosp. Bull. 1947, **81**, 245.
3. Grob, D., Harvey, A. M., Langworthy, O. R., and Lilienthal, J. L., Jr. The administration of di-isopropyl fluorophosphate (DFP) to man. III. Effect on the central nervous system with special reference to the electrical activity of the brain. Johns Hopk. Hosp. Bull. 1947, **81**, 257.
4. Harvey, A. M., Lilienthal, J. L., Jr., Grob, D., Jones, B. F., and Talbot, S. A. The administration of di-isopropyl fluorophosphate to man. IV. The effects on neuromuscular function in normal subjects and in myasthenia gravis. Johns Hopk. Hosp. Bull. 1947, **81**, 267.
5. Leopold, I. H., and Comroe, J. H., Jr. Use of di-isopropyl fluorophosphate ("DFP") in treatment of glaucoma. Arch. Ophthal. (Chicago) 1946, **36**, 1.
6. Grob, D., and Harvey, A. M. Observations on the effects of tetraethyl pyrophosphate (TEPP) in man, and on its use in the treatment of myasthenia gravis. Johns Hopk. Hosp. Bull. 1949, **84**, 532.
7. Rider, J. A., Schulman, S., Richter, R. B., Moeller, H. C., and DuBois, K. P. Treatment of myasthenia gravis with octamethyl pyrophosphoramide: Preliminary report. J. Amer. med. Ass. 1951, **145**, 967.
8. Grob, D., Garlick, W. L., and Harvey, A. M. The toxic effects in man of the anticholinesterase insecticide parathion (p-nitrophenyl diethyl thionophosphate). Johns Hopk. Hosp. Bull. 1950, **87**, 106.
9. Pharmacology and Toxicology of Certain Organic Phosphorus Insecticides, Report to the Council on Pharmacy and Chemistry of the Amer. Med. Ass. J. Amer. Med. Ass. 1950, **144**, 104.
10. Grob, D. Uses and hazards of the organic phosphate anticholinesterase compounds. Ann. intern. Med. 1950, **32**, 1229.
11. Wood, J. R. Chemical defense. J. Amer. med. Ass. 1951, **145**, 1264.
12. Grob, D., and Harvey, A. M. The effects and treatment of nerve gas poisoning. Amer. J. Med. 1953, **14**, 52.
13. Grob, D. The manifestations and treatment of poisoning due to nerve gas and other organic phosphate anticholinesterase compounds. Arch. intern. Med. 1956, **98**, 221.
14. Grob, D. Manifestations and treatment of nerve gas poisoning in man. U. S. armed forces med. J. 1956, **7**, 781.

15. Krop, S., and Kunkel, A. M. Observations on pharmacology of the anticholinesterases sarin and tabun. Proc. Soc. exp. Biol. (N. Y.) 1954, **86**, 530.
16. Holmstedt, B. Synthesis and pharmacology of dimethylamido-ethoxy-phosphoryl cyanide (tabun) together with a description of some allied anticholinesterase compounds containing the N-P bond. Acta physiol. scand. 1951, **25**, Suppl. **90**, 1.
17. Gehauf, B., Epstein, J., Wilson, G. B., Witten, B., Sass, S., Bauer, V. E., and Rueggeberg, W. H. C. Reaction for colorimetric estimation of some phosphorus compounds. Analyt. Chem. 1957, **29**, 278.
18. Michel, H. O. An electrometric method for the determinatioin of red blood cell and plasma cholinesterase activity. J. Lab. clin. Med. 1949, **34**, 1564.
19. Grob, D. The anticholinesterase activity in vitro of the insecticide parathion (p-nitrophenyl diethyl thionophosphate). Johns Hopk. Hosp. Bull. 1950, **87**, 95.
20. Goldstein, A., Krayer, O., Root, M. A., Acheson, G. H., and Doherty, M. E. Plasma neostigmine levels and cholinesterase inhibition in dogs and myasthenic patients. J. Pharmacol. exp. Ther. 1949, **96**, 56.
21. Askew, B. M. Oximes and hydroxamic acids as antidotes in anticholinesterase poisoning. Brit. J. Pharmacol. 1956, **11**, 417.
22. Wilson, I. B., and Sondheimer, F. A specific antidote against lethal alkyl phosphate intoxication. V. Antidotal properties. Arch. Biochem. 1957, **69**, 468.
23. Mangun, G. H., and DuBois, K. P. Toxicity and mechanism of action of tetraethyl pyrophosphate. Fed. Proc. 1947, **6**, 353.
24. Horton, R. G., Koelle, G. B., McNamara, B. P., and Pratt, H. J. The acute toxicity of di-isopropyl fluorophosphate. J. Pharmacol. exp. Ther. 1946, **87**, 414.
25. DuBois, K. P., Doull, J., Salerno, P. R., and Coon, J. M. Studies on the toxicity and mechanism of action of p-nitrophenyl diethyl thionophosphate (parathion). J. Pharmacol. exp. Ther. 1949, **95**, 79.
26. Diggle, W. M., and Gage, J. C. Cholinesterase inhibition *in vitro* by O O-diethyl O-P-nitrophenyl thiophosphate (parathion, E 605). Biochem. J. 1951, **49**, 491.
27. Mazur, A. An enzyme in animal tissues capable of hydrolyzing the phosphorus-fluorine bond of alkyl fluorophosphates. J. biol. Chem. 1946, **164**, 271.
28. Grob, D. Cholinesterase activity of the tissues of man determined post mortem: "Normal" values in infants and adults, and values in liver disease, myasthenia gravis, and after exposure to anticholinesterase compounds. Quart. Progress Report to Chemical Corps Medical Laboratories, Oct. 1, 1949.
29. Riker, W. F., and Wescoe, W. C. The relationship between cholinesterase inhibition and function in a neuroeffector system. J. Pharmacol. exp. Ther. 1949, **95**, 515.
30. Wilson, I. B., and Ginsburg, S. Reactivation of acetylcholinesterase inhibited by alkylphosphates. Arch. Biochem. 1955, **54**, 569.
31. Grob, D., and Johns, R. J. Use of oximes in the treatment of intoxication by anticholinesterase compounds in normal subjects. Amer. J. Med. 1958. In press.
32. Grob, D., and Johns, R. J. Use of oximes in the treatment of intoxication by anticholinesterase compounds in patients with myasthenia gravis. Amer. J. Med. 1958. In press.

# THE INFLUENCE OF BLOOD GLUCOSE ON THE RENAL CLEARANCE OF PHOSPHATE[1, 2]

By E. R. HUFFMAN, C. J. HLAD, Jr., N. E. WHIPPLE, and H. ELRICK

*(From the Medical and Radioisotope Services, Veterans Administration Hospital, and the Department of Medicine, University of Colorado School of Medicine, Denver, Colo.)*

(Submitted for publication July 19, 1957; accepted November 1, 1957)

Many factors have been shown to influence the level of inorganic phosphorus [3] in the blood (1–5). Among these are glucose and insulin, each of which consistently decreases plasma phosphate (1, 3). Since it has been shown that phosphate excretion is dependent upon plasma phosphate (5), one would expect insulin or glucose to alter phosphate excretion in a predictable manner. However, reports on this point have been very conflicting; decreases (6), increases (1, 7) and no change (8, 9) in phosphate excretion following glucose or insulin have been described.

During the course of studies on the renal effects of glucagon (10, 11), a consistent relationship between blood glucose level and renal phosphate clearance was observed. The purpose of this paper is to define this relationship and to point out its physiologic implications.

## METHODS

Studies were carried out in 40 hospitalized ambulatory male patients with no evidence of renal disease. All subjects were studied in the postabsorptive state (14 hour fast). Water was ingested before and during the experiments to assure adequate urine flows. Intravenous infusion of glucose, insulin,[4] or buffered phosphate (pH 7.4) solutions was accomplished by a Bowman constant infusion pump. In two subjects glucose was administered orally as a 50 per cent solution. In two other subjects tolbutamide (Orinase®, sodium)[5] was administered as a single intravenous injection over a five minute period. Standard renal clearance techniques were used with endogenous creatinine clearance as a measure of glomerular filtration rate. Phosphate clearance is expressed as percentage of filtered load excreted, *i.e.*, phosphate clearance times 100 divided by creatinine clearance. All clearance data are corrected to 1.73 square meters of body surface area.

Urine was collected over 10 or 15 minute periods through a multiholed, indwelling catheter and complete collections were assured by multiple water and air washouts. Heparinized blood samples were obtained from the antecubital vein through an indwelling needle at the midpoint of each collection period. The samples were centrifuged immediately and plasma removed for analysis.

Creatinine determinations were made by the method of Bonsnes and Taussky (12). The Nelson-Somogyi method was used for blood glucose determinations (13). Urinary glucose was measured by the Benedict quantitative method (14). Phosphate was determined by the method of Fiske and Subbarow (15).

## RESULTS

### *I. Effect of glucose on phosphate clearance*

Eighteen subjects received glucose by vein or mouth. Blood sugar was elevated gradually in some, rapidly in others. In all subjects the effect on phosphate excretion was similar. One of these experiments is shown in Figure 1. Note the parallel relationship between blood sugar and phosphate clearance (expressed as percentage of filtered load excreted [6]). The pertinent data on all subjects are given in Table I. Creatinine clearance was not affected by glucose loading. In 16 of the 18 subjects there was a significant positive correlation ($R_G$)[7] between blood sugar and phosphate

[1] Presented at a meeting of the American Federation for Clinical Research, Carmel, Calif., January 30, 1957.

[2] Supported in part by a grant from the Upjohn Co., Kalamazoo, Mich.

[3] Hereafter, inorganic phosphorous is referred to as phosphate.

[4] Insulin free of glucagon (Lot T-3206) kindly supplied by Dr. W. R. Kirtley, of Eli Lilly and Co.

[5] Kindly supplied by Dr. C. J. O'Donovan, the Upjohn Co.

[6] The following abbreviations will be used in this report: $C_P$, renal clearance of phosphate; $C_C$, renal clearance of endogenous creatinine; $P_P$, plasma inorganic phosphate; $R_G$, correlation coefficient relating blood glucose and the percentage of filtered load of phosphate excreted; $R_P$, correlation coefficient relating plasma phosphate and the percentage of filtered load of phosphate excreted; p, the probability (indicative of significance level) associated with the correlation coefficients.

[7] Since the experimental data indicated linearity between blood sugar and phosphate clearance in the majority of cases, the linear correlation coefficient was used as an index of the strength of relationship.

TABLE I

*Effect of blood glucose on phosphate clearance*

| Subject | Glucose administration | Time* | $C_C$† | $P_P$‡ | $C_P/C_C$§ | Blood or plasma sugar | $R_G$‖ | p¶ | $R_P$‖ | p¶ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *min.* | *ml./min.* | *mg. %* | % | *mg. %* | | | | |
| C. M. | | 0 | 97 | 2.86 | 6.1 | 92 | 0.97 | <0.001 | 0.01 | >0.10 |
| 37 yrs. | 398 mg./min. | | | | | | | | | |
| 63 Kg. | I.V. | 20.2 | 91 | 3.10 | 8.7 | 106 | | | | |
| | | 40.8 | 96 | 3.02 | 13.3 | 145 | | | | |
| | | ↓61.8 | 96 | 2.79 | 14.6 | 167 | | | | |
| J. L. | | 0 | 93 | 3.31 | 9.8 | 85 | 0.96 | <0.001 | −0.66 | 0.02 |
| 54 yrs. | 440 mg./min. | | | | | | | | | |
| 67 Kg. | I.V. | 23.0 | 93 | 3.29 | 13.7 | 102 | | | | |
| | | 43.0 | 93 | 3.20 | 18.1 | 140 | | | | |
| | | 64.0 | 97 | 3.11 | 21.2 | 160 | | | | |
| | | 89.0 | 95 | 3.11 | 22.6 | 162 | | | | |
| | | ↓99.0 | 91 | 3.02 | 18.7 | 134 | | | | |
| A. T. | | 0 | 119 | 2.60 | 5.4 | 72 | 0.96 | <0.001 | −0.31 | >0.10 |
| 32 yrs. | 474 mg./min. | | | | | | | | | |
| 62 Kg. | I.V. | 19.5 | 118 | 2.79 | 6.2 | 83 | | | | |
| | | 37.7 | 108 | 2.78 | 9.7 | 107 | | | | |
| | | 57.7 | 98 | 2.82 | 14.4 | 144 | | | | |
| | | ↓68.7 | 110 | 2.87 | 13.4 | 160 | | | | |
| J. M. | | 0 | 94 | 2.55 | 11.9 | 67 | 0.94 | <0.001 | −0.53 | 0.10 |
| 37 yrs. | 150 Gm. ——→ | | | | | | | | | |
| 80 Kg. | Oral | 20.1 | 98 | 2.63 | 16.0 | 81 | | | | |
| | | 40.2 | 92 | 2.64 | 29.2 | 129 | | | | |
| | | 60.1 | 76 | 2.39 | 32.6 | 142 | | | | |
| | | 79.9 | 89 | 2.27 | 30.9 | 130 | | | | |
| A. W. | | 0 | 115 | 3.58 | 20.7 | 87 | 0.82 | <0.01 | −0.42 | >0.10 |
| 31 yrs. | 908 mg./min. | | | | | | | | | |
| 79 Kg. | I.V. | 20.5 | 113 | 3.48 | 25.3 | 105 | | | | |
| | | 40.0 | 101 | 3.25 | 37.2 | 162 | | | | |
| | | 60.9 | 103 | 3.29 | 34.3 | 192 | | | | |
| | | ↓79.1 | 95 | 4.14 | 32.4 | 210 | | | | |
| T. T. | | 0 | 107 | 2.92 | 9.0 | 70 | 0.81 | <0.01 | −0.42 | >0.10 |
| 32 yrs. | 475 mg./min. | | | | | | | | | |
| 56 Kg. | I.V. | 19.2 | 123 | 2.89 | 10.8 | 93 | | | | |
| | | 39.7 | 123 | 2.73 | 13.0 | 128 | | | | |
| | | ↓59.6 | 123 | 2.75 | 14.3 | 138 | | | | |
| | | 80.2 | 114 | 2.75 | 11.3 | 122 | | | | |
| | | 89.5 | 124 | 2.71 | 6.7 | 64 | | | | |
| L. Q. | | 0 | 105 | 2.89 | 17.9 | 74 | 0.80 | <0.001 | −0.44 | >0.10 |
| 26 yrs. | 547 mg./min. | | | | | | | | | |
| 64 Kg. | I.V. | 19.2 | 107 | 2.81 | 21.3 | 84 | | | | |
| | | 39.3 | 108 | 2.81 | 23.9 | 100 | | | | |
| | | 59.0 | 107 | 2.75 | 24.3 | 155 | | | | |
| | | ↓80.5 | 102 | 2.61 | 29.4 | 177 | | | | |
| | | 100.5 | 103 | 2.59 | 24.9 | 162 | | | | |
| | | 109.2 | 104 | 2.53 | 19.9 | 140 | | | | |
| A. J. | | 0 | 109 | 4.09 | 8.0 | 67 | 0.79 | <0.01 | −0.84 | <0.001 |
| 30 yrs. | 1,062 mg./min. | | | | | | | | | |
| 69 Kg. | I.V. | 21.2 | 113 | 3.83 | 13.8 | 111 | | | | |
| | | 42.2 | 105 | 3.50 | 30.7 | 193 | | | | |
| | | 62.3 | 111 | 3.13 | 29.0 | 258 | | | | |
| | | 83.7 | 109 | 3.12 | 25.1 | 332 | | | | |
| | | ↓90.7 | 98 | 3.01 | 23.8 | 302 | | | | |

* For the sake of brevity, control values are the mean of at least three 10 minute clearance periods, and all 20 minute periods are the mean values of two 10 minute periods.

† $C_C$, creatinine clearance.

‡ $P_P$, plasma phosphate.

§ $C_P/C_C$, per cent of filtered load of phosphate excreted.

‖ $R_G$, correlation coefficient relating phosphate clearance and blood glucose; $R_P$, correlation coefficient relating phosphate clearance and plasma phosphate. Correlation coefficients are calculated from the 10 minute period values.

¶ p, significance level.

TABLE I—*Continued*

| Subject | Glucose administration | Time* | $C_C$† | $P_p$‡ | $C_P/C_C$§ | Blood or plasma sugar | $R_G$‖ | $_pP$¶ | $R_P$‖ | $_pP$¶ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *min.* | *ml./min.* | *mg. %* | % | *mg. %* | | | | |
| A. C. | | 0 | 114 | 2.91 | 8.6 | 62 | 0.93 | <0.001 | −0.72 | 0.02 |
| 29 yrs. | 878 mg./min. | | | | | | | | | |
| 79 Kg. | I.V. | 21.2 | 121 | 2.87 | 12.8 | 64 | | | | |
| | | 42.8 | 108 | 2.80 | 22.3 | 128 | | | | |
| | | 64.7 | 107 | 2.87 | 26.9 | 176 | | | | |
| | | 75.3 | 111 | 2.83 | 23.7 | 200 | | | | |
| M. A. | | 0 | 98 | 2.45 | 9.5 | 89 | 0.92 | <0.001 | −0.46 | >0.10 |
| 49 yrs. | 100 Gm. ⟶ | | | | | | | | | |
| 79 Kg. | Oral | 20.8 | 101 | 2.44 | 11.7 | 90 | | | | |
| | | 41.1 | 100 | 2.28 | 22.0 | 132 | | | | |
| | | 61.5 | 103 | 2.20 | 22.8 | 143 | | | | |
| | | 81.2 | 105 | 2.08 | 18.6 | 140 | | | | |
| M. P. | | 0 | 130 | 3.70 | 6.9 | 87 | 0.90 | 0.001 | −0.80 | 0.02 |
| 41 yrs. | 472 mg./min. | | | | | | | | | |
| 67 Kg. | I.V. | 20.6 | 121 | 3.69 | 8.9 | 109 | | | | |
| | | 40.3 | 128 | 3.38 | 10.0 | 127 | | | | |
| | | 50.8 | 128 | 3.36 | 9.4 | 137 | | | | |
| R. G. | | 0 | 132 | 3.26 | 11.9 | 100 | 0.89 | <0.01 | −0.50 | >0.10 |
| 28 yrs. | 15 Gm. ⟶ | | | | | | | | | |
| 82 Kg. | I.V. | 28.1 | 148 | 3.10 | 18.0 | 163 | | | | |
| | | 56.9 | 121 | 3.31 | 11.1 | 112 | | | | |
| | | 71.2 | 138 | | | | | | | |
| W. N. | | 0 | 121 | 3.15 | 13.5 | 69 | 0.85 | <0.01 | −0.71 | 0.02 |
| 26 yrs. | 694 mg./min. | | | | | | | | | |
| 67 Kg. | I.V. | 19.1 | 127 | 3.18 | 20.3 | 101 | | | | |
| | | 40.3 | 125 | 2.94 | 27.8 | 142 | | | | |
| | | 59.2 | 127 | 2.82 | 24.9 | 178 | | | | |
| | | 70.0 | 120 | 2.66 | 26.7 | 188 | | | | |
| K. Q. | | 0 | 128 | 2.76 | 6.6 | 69 | 0.75 | <0.01 | −0.52 | 0.08 |
| 51 yrs. | 477 mg./min. | | | | | | | | | |
| 78 Kg. | I.V. | 24.9 | 133 | 2.96 | 6.8 | 93 | | | | |
| | | 46.2 | 130 | 2.82 | 9.7 | 110 | | | | |
| | | 69.5 | 127 | 2.53 | 10.7 | 111 | | | | |
| | | 89.4 | 135 | 2.88 | 8.0 | 102 | | | | |
| | | 101.3 | 125 | 3.20 | 4.2 | 85 | | | | |
| C. R. | | 0 | 78 | 3.91 | 28.7 | 85 | 0.73 | <0.01 | −0.24 | >0.10 |
| 31 yrs. | 507 mg./min. | | | | | | | | | |
| 86 Kg. | I.V. | 20.5 | 79 | 3.97 | 30.8 | 100 | | | | |
| | | 41.4 | 84 | 3.55 | 34.8 | 127 | | | | |
| | | 63.3 | 81 | 3.54 | 35.9 | 135 | | | | |
| | | 83.7 | 82 | 3.36 | 31.4 | 123 | | | | |
| | | 103.4 | 78 | 3.17 | 29.1 | 81 | | | | |
| C. C. | | 0 | 93 | 2.60 | 3.9 | 70 | 0.57 | 0.06 | −0.70 | 0.015 |
| 44 yrs. | 325 mg./min. | | | | | | | | | |
| 82 Kg. | I.V. | 19.9 | 99 | 2.59 | 3.4 | 82 | | | | |
| | | 40.8 | 99 | 2.60 | 5.7 | 117 | | | | |
| | | 61.3 | 93 | 2.38 | 5.6 | 124 | | | | |
| | | 81.6 | 95 | 2.75 | 2.8 | 102 | | | | |
| J. L. | | 0 | 94 | 2.80 | 18.2 | 67 | 0.41 | >0.10 | −0.61 | 0.05 |
| 39 yrs. | 1,040 mg./min. | | | | | | | | | |
| 64 Kg. | I.V. | 17.6 | 104 | 2.42 | 30.8 | 138 | | | | |
| | | 39.4 | 81 | 2.26 | 39.7 | 229 | | | | |
| | | 58.8 | 79 | 2.08 | 34.6 | 315 | | | | |
| | | 78.5 | 71 | 2.14 | 28.4 | 382 | | | | |
| | | 88.3 | 61 | 1.89 | 28.8 | 409 | | | | |
| D. L. | | 0 | 126 | 2.60 | 7.1 | 74 | 0.24 | >0.10 | −0.49 | >0.10 |
| 23 yrs. | 942 mg./min. | | | | | | | | | |
| 84 Kg. | I.V. | 19.7 | 128 | 2.54 | 9.1 | 93 | | | | |
| | | 40.4 | 125 | 2.48 | 11.6 | 146 | | | | |
| | | 60.8 | 114 | 2.34 | 11.1 | 175 | | | | |
| | | 79.7 | 121 | 2.37 | 9.0 | 196 | | | | |
| | | 100.1 | 119 | 2.52 | 6.9 | 167 | | | | |

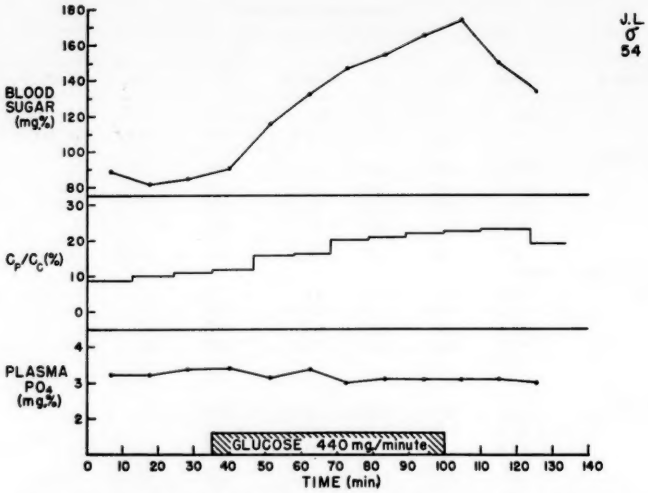



FIG. 1. THE EFFECT OF BLOOD GLUCOSE ON PHOSPHATE CLEARANCE

During the infusion of glucose, phosphate clearance parallels blood glucose but only a slight decrease in plasma phosphate occurs.

clearance. In the remaining two subjects, significant positive correlations were maintained until a maximum level of phosphate clearance was reached (*vide infra*).

In contrast, there was a negative correlation ($R_P$) [8] in all but one subject between plasma phosphate and phosphate clearance. This negative correlation was insignificant in 16 of the 18 experiments.

In eight subjects phosphate clearance reached a maximum level despite a progressive increase in blood sugar levels. This phenomenon was a consistent observation in the four subjects who manifested glycosuria. The maximum level of phosphate clearance bore no apparent relationship to blood glucose level and was extremely variable from patient to patient.

### *II. Effect of insulin and tolbutamide on phosphate clearance*

Blood sugar was lowered in five subjects with insulin or tolbutamide (Orinase®, sodium). In no instance did symptoms or signs of hypoglycemia develop despite marked blood sugar depression. Creatinine clearances did not change significantly. Figure 2 shows the data from one of the experiments. The results of all these experi-

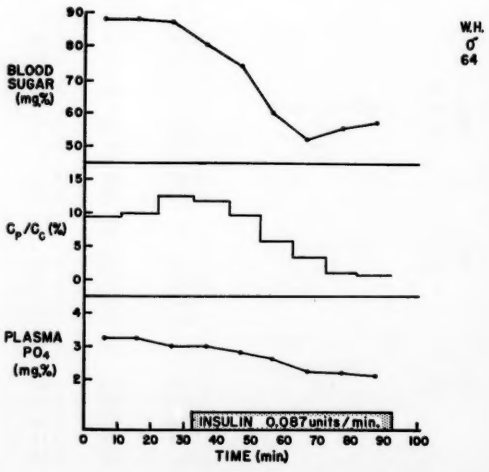



FIG. 2. THE EFFECT OF INSULIN ON PHOSPHATE CLEARANCE

During the infusion of insulin, phosphate clearance, blood sugar and plasma phosphate behave in a parallel manner.

[8] Experimental data indicated linearity between plasma phosphate and phosphate clearance over the limited range studied. Hence, the linear correlation coefficient was employed.

TABLE II

*Effect of insulin and tolbutamide (Orinase®) on phosphate clearance* *

| Subject | Intravenous injections | Time | $C_C$ | $P_P$ | $C_P/C_C$ | Blood or plasma sugar | $R_G$ | p | $R_P$ | p |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *min.* | *ml./min.* | *mg. %* | *%* | *mg. %* | | | | |
| W. H. | | 0 | 89 | 3.19 | 10.5 | 88 | 0.91 | <0.001 | 0.87 | 0.001 |
| 64 yrs. | Insulin † | | | | | | | | | |
| 44 Kg. | 0.093 unit/min. | 20.0 | 103 | 2.93 | 10.7 | 78 | | | | |
| | | 39.7 | 95 | 2.45 | 3.3 | 56 | | | | |
| | | 59.6 | 98 | 2.17 | 0.8 | 57 | | | | |
| F. M. | | 0 | 113 | 3.08 | 10.0 | 87 | 0.99 | <0.001 | 0.82 | <0.01 |
| 46 yrs. | Insulin | | | | | | | | | |
| 67 Kg. | 0.10 unit/min. | 20.2 | 120 | 3.09 | 9.9 | 84 | | | | |
| | | 40.9 | 124 | 2.81 | 6.3 | 70 | | | | |
| | | 60.0 | 123 | 2.74 | 2.3 | 52 | | | | |
| L. S. | | 0 | 109 | 3.34 | 13.0 | 94 | 0.93 | <0.001 | 0.94 | <0.001 |
| 30 yrs. | Insulin | | | | | | | | | |
| 67 Kg. | 0.096 unit/min. | 18.9 | 111 | 3.25 | 13.7 | 90 | | | | |
| | | 41.0 | 113 | 3.07 | 8.0 | 64 | | | | |
| | | 61.0 | 118 | 2.72 | 5.1 | 53 | | | | |
| L. H. | | 0 | 120 | 3.16 | 8.5 | 107 | 0.94 | <0.001 | 0.95 | <0.001 |
| 30 yrs. | Orinase® ——→ | | | | | | | | | |
| 75 Kg. | 1.5 Gm. | 36.6 | 116 | 2.89 | 2.6 | 70 | | | | |
| | | 56.6 | 123 | 2.75 | 1.3 | 66 | | | | |
| | | 66.1 | 138 | 2.78 | 1.7 | 77 | | | | |
| R. R. | | 0 | 123 | 2.80 | 2.6 | 87 | 0.64 | 0.05 | 0.68 | 0.05 |
| 22 yrs. | Orinase® ——→ | | | | | | | | | |
| 73 Kg. | 2.0 Gm. | 21.2 | 120 | 2.37 | 3.1 | 65 | | | | |
| | | 42.0 | 134 | 1.73 | 0.5 | 42 | | | | |
| | | 61.9 | 140 | 1.55 | 0.5 | 67 | | | | |
| R. M. | Glucose——→ | | | | | | | | | |
| 21 yrs. | Load, 8 Gm. | 0 | 135 | 2.15 | 5.5 | 171 | 0.92 | <0.001 | 0.68 | 0.015 |
| 71 Kg. | Infusion, | 9.7 | 135 | 1.60 | 4.7 | 162 | | | | |
| | 336 mg./min. | 19.6 | 125 | 1.56 | 1.9 | 157 | | | | |
| | | 29.2 | 135 | 1.49 | 2.5 | 143 | | | | |
| | Insulin | 39.8 | 122 | 1.44 | 1.6 | 121 | | | | |
| | 0.29 unit/min. | 49.6 | 130 | 1.31 | 1.2 | 107 | | | | |
| | | 59.6 | 128 | 1.51 | 0.1 | 98 | | | | |
| | | 69.8 | 141 | 1.54 | 0.7 | 91 | | | | |
| T. C. | Glucose——→ | | | | | | | | | |
| 43 yrs. | Load, 12 Gm. | 0 | 99 | 2.44 | 22.1 | 165 | 0.96 | <0.001 | 0.97 | <0.001 |
| 89 Kg. | Infusion, | 9.6 | 110 | 2.20 | 20.5 | 151 | | | | |
| | 331 mg./min. | 19.6 | 122 | 2.17 | 17.0 | 147 | | | | |
| | | 29.0 | 116 | 2.09 | 16.4 | 140 | | | | |
| | Insulin | 39.4 | 126 | 2.00 | 11.7 | 135 | | | | |
| | 0.17 unit/min. | 49.0 | 134 | 1.92 | 8.0 | 131 | | | | |
| | | 59.4 | 126 | 1.70 | 4.5 | 128 | | | | |
| | | 69.4 | 126 | 1.58 | 3.0 | 119 | | | | |
| | | 79.4 | 127 | 1.52 | 2.2 | 118 | | | | |
| K. W. | | 0 | 105 | 3.43 | 17.3 | 90 | 0.91 | <0.001 | −0.03 | >0.10 |
| 28 yrs. | Glucose——→ | | | | | | | | | |
| 63 Kg. | Load, 16 Gm. | 10.8 | 88 | 3.11 | 24.3 | 186 | | | | |
| | Infusion, | 21.1 | 116 | 2.97 | 23.2 | 186 | | | | |
| | 349 mg./min. | 31.9 | 105 | 2.99 | 22.9 | 186 | | | | |
| | Insulin | 42.3 | 110 | 2.94 | 20.5 | 176 | | | | |
| | 0.14 unit/min. | 51.7 | 106 | 2.86 | 20.8 | 167 | | | | |
| | | 62.2 | 113 | 2.83 | 18.7 | 137 | | | | |
| | | 72.3 | 109 | 2.90 | 16.7 | 131 | | | | |

* For definitions, see Table I.

† Heavy vertical arrows refer to the insulin infusion period.

TABLE II—*Continued*

| Subject | Intravenous injections | Time | Cc | Pp | Cp/Cc | Blood or plasma sugar | Rg | p | Rp | p |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *min.* | *ml./min.* | *mg. %* | *%* | *mg. %* | | | | |
| G. B. | | 0 | 127 | 3.12 | 4.7 | 89 | 0.91 | <0.001 | 0.01 | >0.10 |
| 38 yrs. | Glucose→ | | | | | | | | | |
| 61 Kg. | Load, 16 Gm. | 22.3 | 116 | 2.91 | 27.5 | 179 | | | | |
| | Infusion, | 33.0 | 121 | 2.70 | 25.0 | 195 | | | | |
| | 352 mg./min. | 44.8 | 128 | 2.61 | 13.8 | 173 | | | | |
| | Insulin | 56.7 | 132 | 2.48 | 15.0 | 168 | | | | |
| | 0.077 unit/min. | 67.4 | 124 | 2.36 | 12.8 | 145 | | | | |
| | | 80.1 | 118 | 2.25 | 9.8 | 125 | | | | |
| | | 91.4 | 136 | 2.19 | 10.2 | 114 | | | | |
| | | 102.0 | 129 | 2.26 | 4.9 | 114 | | | | |
| | | ↓111.7 | 136 | 2.12 | 3.8 | 104 | | | | |
| C. S. | | 0 | 94 | 3.38 | 13.6 | 81 | 0.43 | >0.10 | 0.10 | >0.10 |
| 35 yrs. | Glucose→ | | | | | | | | | |
| 67 Kg. | Load, 8 Gm. | 11.2 | 96 | 3.43 | 21.8 | 105 | | | | |
| | Infusion, | 21.7 | 96 | 3.14 | 19.7 | 113 | | | | |
| | 185 mg./min. | 30.7 | 93 | 3.29 | 16.1 | 113 | | | | |
| | Insulin | 41.7 | 94 | 2.93 | 16.4 | 127 | | | | |
| | 0.24 unit/min. | 51.0 | 98 | 2.90 | 15.2 | 116 | | | | |
| | | 62.0 | 96 | 2.92 | 14.3 | 109 | | | | |
| | | ↓72.4 | 98 | 2.82 | 14.6 | 101 | | | | |

ments are given in Table II. Note that in every case there was a simultaneous decrease in blood glucose, plasma phosphate and phosphate clearance following the control periods. Thus, there was a significant positive correlation between blood sugar and phosphate clearance as well as between plasma phosphate and phosphate clearance.

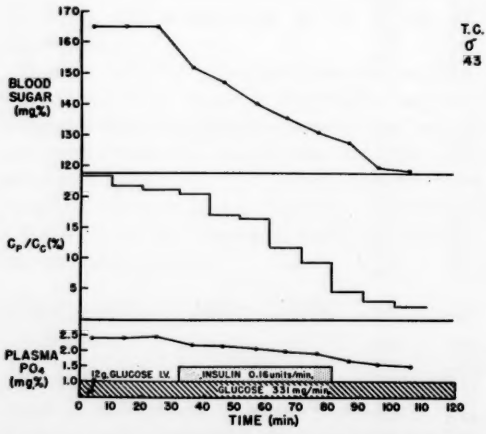



FIG. 3. THE EFFECT OF GLUCOSE AND INSULIN ON PHOSPHATE CLEARANCE

Phosphate clearance and blood glucose curves are remarkably parallel when blood glucose is lowered by insulin from hyperglycemic levels.

*III. Effect of glucose and insulin on phosphate clearance*

Five subjects were given glucose followed by insulin. Figure 3 shows the data from one experiment. The results from all these experiments are given in Table II. As in the experiments with glucose alone, phosphate clearance paralleled blood glucose, and significant positive correlations between blood sugar and phosphate clearance were present in four of the five subjects tested. In the remaining subject the correlation was positive but insignificant. Due to a continuous decrease in the plasma phosphate and an initial increase in phosphate clearance (resulting from the glucose load), correlations between plasma phosphate and phosphate clearance were poor. However, this correlation improved following insulin administration.

*IV. Effect of phosphate on phosphate clearance*

In nine subjects plasma phosphate was elevated by phosphate administration. These results are given in Table III. The correlation between plasma phosphate and phosphate clearance was excellent in all experiments. In two subjects (J. C. and J. P.) plasma phosphate was elevated gradually; in the remaining subjects, it was elevated rapidly. The results of the former experiments are shown in Figure 4. In the group of subjects

TABLE III

*Effect of plasma phosphate on phosphate clearance* *

| Subject | $PO_4$ inj., I.V. | Time | $C_C$ | $P_P$ | $C_P/C_C$ | Blood or plasma sugar | $R_P$ | p | $R_G$ | p |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *min.* | *ml./min.* | *mg. %* | *%* | *mg. %* | | | | |
| R. J. | | 0 | 67 | 2.52 | 12.7 | 75 | 0.87 | <0.001 | 0.81 | <0.001 |
| 58 yrs. | 221 mg. → | | | | | | | | | |
| 87 Kg. | 6.2 mg./min. | 21.9 | 75 | 4.20 | 61.3 | 82 | | | | |
| | | 40.7 | 79 | 4.72 | 54.9 | 84 | | | | |
| | | ↓66.9 | 76 | 5.17 | 51.2 | 84 | | | | |
| E. S. | | 0 | 98 | 3.29 | 6.6 | 81 | 0.99 | <0.001 | 0.76 | 0.02 |
| 36 yrs. | 221 mg. → | | | | | | | | | |
| 57 Kg. | 6.0 mg./min. | 21.0 | 102 | 6.21 | 45.4 | 85 | | | | |
| | | 43.9 | 100 | 6.45 | 46.8 | 90 | | | | |
| | | ↓64.8 | 104 | 6.88 | 46.9 | 93 | | | | |
| R. L. | | 0 | 135 | 3.33 | 14.1 | 90 | 0.86 | <0.001 | −0.41 | >0.10 |
| 32 yrs. | 190 mg. → | | | | | | | | | |
| 61 Kg. | 5.2 mg./min. | 19.6 | 133 | 4.88 | 24.1 | 85 | | | | |
| | | 40.6 | 143 | 5.23 | 21.2 | 80 | | | | |
| | | ↓62.6 | 133 | 5.47 | 22.8 | 91 | | | | |
| L. A. | | 0 | 130 | 3.09 | 6.2 | 95 | 0.98 | <0.001 | −0.37 | >0.10 |
| 37 yrs. | 158 mg. → | | | | | | | | | |
| 75 Kg. | 5.1 mg./min. | 21.4 | 129 | 4.73 | 28.7 | 91 | | | | |
| | | 41.5 | 128 | 5.44 | 31.4 | 94 | | | | |
| | | ↓52.4 | 119 | 5.48 | 40.4 | 93 | | | | |
| L. O. | | 0 | 117 | 2.84 | 4.3 | 92 | 0.99 | <0.001 | −0.18 | >0.10 |
| 38 yrs. | 158 mg. → | | | | | | | | | |
| 81 Kg. | 5.8 mg./min. | 21.0 | 119 | 4.51 | 28.8 | 91 | | | | |
| | | 44.8 | 122 | 4.95 | 31.3 | 93 | | | | |
| | | ↓55.9 | 122 | 5.22 | 34.3 | 92 | | | | |
| R. M. | | 0 | 130 | 1.90 | 7.0 | 80 | 0.98 | <0.001 | 0.21 | >0.10 |
| 30 yrs. | 158 mg. → | | | | | | | | | |
| 92 Kg. | 10.0 mg./min. | 21.2 | 134 | 3.74 | 34.3 | 79 | | | | |
| | | 42.1 | 138 | 4.20 | 46.6 | 79 | | | | |
| | | ↓51.8 | 143 | 4.83 | 47.4 | 88 | | | | |
| J. N. | | 0 | 127 | 2.82 | 8.9 | 71 | 0.97 | <0.001 | 0.29 | >0.10 |
| 24 yrs. | 285 mg. → | | | | | | | | | |
| 79 Kg. | 5.3 mg./min. | 20.2 | 129 | 5.59 | 42.6 | 70 | | | | |
| | | 41.8 | 126 | 5.56 | 38.4 | 76 | | | | |
| | | ↓60.7 | 144 | 5.67 | 38.7 | 80 | | | | |
| J. C. | | 0 | 91 | 2.38 | 13.4 | 94 | 0.97 | <0.001 | 0.65 | 0.05 |
| 59 yrs. | 0 mg. → | | | | | | | | | |
| 71 Kg. | 11.0 mg./min. | 23.5 | 105 | 3.03 | 20.4 | 91 | | | | |
| | | 43.5 | 107 | 4.02 | 35.6 | 99 | | | | |
| | | 64.9 | 97 | 4.80 | 42.1 | 101 | | | | |
| | | 86.4 | 100 | 5.63 | 45.1 | 99 | | | | |
| | | ↓97.8 | 93 | 5.91 | 50.3 | 96 | | | | |
| J. P. | | 0 | 102 | 3.11 | 8.4 | 83 | 0.99 | <0.001 | 0.20 | >0.10 |
| 36 yrs. | 0 mg. → | | | | | | | | | |
| 72 Kg. | 10.8 mg./min. | 20.1 | 91 | 3.68 | 21.3 | 82 | | | | |
| | | 40.0 | 92 | 4.78 | 39.0 | 86 | | | | |
| | | ↓59.9 | 101 | 5.20 | 47.6 | 82 | | | | |

* For definitions, see Table I.

in which a priming dose of phosphate was administered, the correlation coefficients were highly significant in all cases despite the excessively high phosphate clearance in the period following the priming dose.

In contrast, the correlation coefficients for blood sugar and phosphate clearance were variable in direction and significance. There were six positive correlations and three negative ones. Only one of the entire group was highly significant.

Creatinine clearances were unaffected by phosphate administration.

### *V. Effect of phosphate and insulin on phosphate clearance*

The data on the three subjects who were given insulin and buffered phosphate solution simultaneously are shown in Table IV. Blood sugar fell in each case as expected. However, plasma phosphate showed a consistent rise rather than the fall which was regularly observed following insulin alone. In all cases the rise in plasma phosphate was accompanied by an increase in phosphate clearance despite the fall in blood glucose.

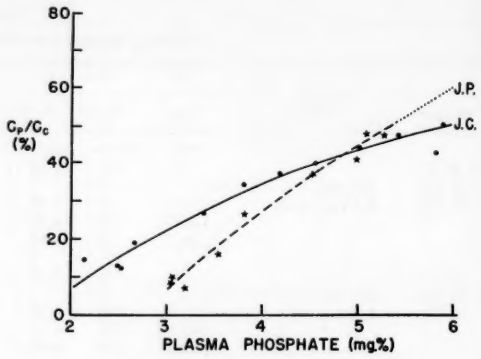



FIG. 4. THE EFFECT OF PLASMA PHOSPHATE ON PHOSPHATE CLEARANCE

Phosphate clearance is shown here as a function of plasma phosphate in two subjects who received a continuous infusion of buffered phosphate solution.

## DISCUSSION

### *Blood glucose and phosphate clearance*

The data show that phosphate clearance consistently parallels the blood glucose level whether it is elevated by glucose administration or depressed by hypoglycemic agents (Figure 5). Thus, the level of venous blood sugar exerts a sensitive and potent influence on phosphate clearance. This influence appears to operate only up to a certain maximum level of phosphate clearance which varies considerably from patient to pa-

TABLE IV

*Effect of phosphate and insulin on phosphate clearance* *

| Subject | Phosphate and insulin infusion, I.V. | Time | $C_C$ | $P_P$ | $C_P/C_C$ | Blood or plasma sugar |
|---|---|---|---|---|---|---|
| | | *min.* | *ml./min.* | *mg. %* | *%* | *mg. %* |
| L. C. | | 9.1 | 118 | 3.06 | 7.0 | 93 |
| 27 yrs. | | 20.0 | 120 | 2.90 | 8.1 | 90 |
| 90.5 Kg. | Phosphate, | 30.0 | 116 | 3.06 | 8.6 | 92 |
| | 11.4 mg./min. † | 40.1 | 128 | 3.79 | 7.1 | 70 |
| | Insulin, | 49.9 | 123 | 3.84 | 11.3 | 62 |
| | 0.11 unit/min. | 60.2 | 121 | 3.84 | 12.7 | 56 |
| D. C. | | 10.4 | 130 | 2.74 | 13.3 | 89 |
| 32 yrs. | | 19.9 | 136 | 2.84 | 11.4 | 88 |
| 88.2 Kg. | Phosphate, | 30.9 | 135 | 2.95 | 11.3 | 87 |
| | 10.4 mg./min. | 40.8 | 129 | 2.94 | 10.2 | 85 |
| | Insulin, | 50.4 | 131 | 3.10 | 17.1 | 83 |
| | 0.13 unit/min. | 60.7 | 122 | 3.26 | 17.0 | 80 |
| | | 74.9 | 127 | 3.39 | 16.0 | 75 |
| | | 85.6 | 126 | 3.63 | 14.0 | 70 |
| | | 96.4 | 132 | 3.92 | 12.6 | 68 |
| P. V. | | 10.7 | 116 | 3.55 | 5.5 | 83 |
| 23 yrs. | | 20.6 | 118 | 3.63 | 5.3 | 85 |
| 65.5 Kg. | | 31.3 | 115 | 3.39 | 5.4 | 87 |
| | Phosphate, | 42.1 | 112 | 3.87 | 6.8 | 87 |
| | 11.0 mg./min. | 50.5 | 99 | 4.19 | 9.8 | 78 |
| | Insulin, | 60.4 | 126 | 4.52 | 9.1 | 67 |
| | 0.11 unit/min. | 71.1 | 124 | 4.42 | 9.6 | 50 |
| | | 83.0 | 122 | 4.42 | 9.1 | 39 |
| | | 90.0 | 106 | 4.66 | 7.9 | 48 |

* For definitions, see Table I.

† Heavy vertical arrows refer to the insulin infusion period.

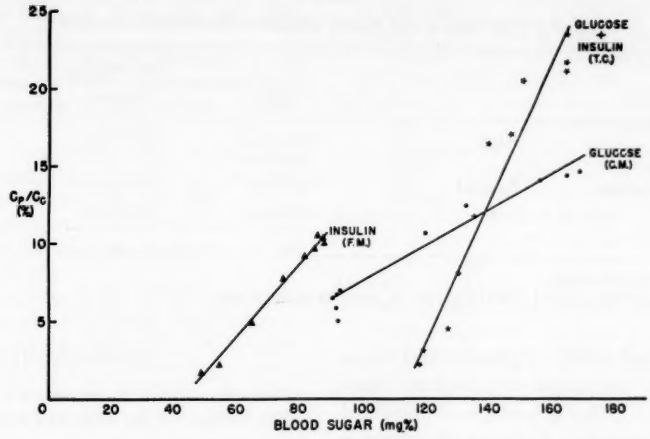



FIG. 5. THE RELATIONSHIP BETWEEN PHOSPHATE CLEARANCE AND BLOOD SUGAR

This graph exemplifies the dependence of phosphate clearance on blood glucose over a wide range of values of the latter.

tient. In all cases of glycosuria, phosphate clearance reached this maximum.

There are a number of reports in the literature which support the concept that phosphate excretion is a direct function of blood sugar level. Levitan (7) reported increased phosphate clearances in normal men following the administration of large amounts of glucose. Unfortunately, his experiments were complicated by the occurrence of glycosuria in all subjects. Reiser (1) also observed increased phosphate excretion in normal men following glucose administration. Pitts and Alexander (16) demonstrated that glucose decreased phosphate reabsorption in the dog when plasma phosphate levels were adequate to show a phosphate Tm (maximal rate of renal tubular reabsorption). They suggested that glucose and phosphate compete for reabsorption. The present data indicate a similar competition at normal or depressed plasma phosphate levels.

Not all previous work substantiates the concept that phosphate excretion is a direct function of blood sugar level. Levenson, Adams, Rosen, and Laskey Taylor (9) found no change in $P^{32}$ excretion following a single injection of $P^{32}$ when glucose or insulin was administered to normal men. Decreases in phosphate excretion following glucose have been reported in the dog by Sokhey and Allan (6). Bachmann, Haldi, Ensor, and Wynn (8) observed no change in urinary inorganic phosphate in the two hour period following ingestion of 50 Gm. of glucose in normal men. These conflicting findings are probably due to the wide variability in experimental methods. It should be pointed out that all studies employing standard renal clearance techniques are in agreement with the present investigation.

*Plasma phosphate and phosphate clearance*

Several laboratories have shown in the dog and man that phosphate excretion was proportional to plasma phosphate over a wide range (16–18). The present experiments show that a direct relationship between plasma phosphate and phosphate clearance exists in man provided blood glucose level does not increase. A rise in blood sugar results in an increased phosphate clearance even though the plasma phosphate usually decreases; hence, a poor correlation between plasma phosphate and phosphate clearance. A fall in blood sugar from hyperglycemic levels is associated with a fair correlation between plasma phosphate and phosphate clearance, whereas a fall in blood sugar below normal fasting levels is associated with an excellent correlation. The simultaneous

TABLE V

*Effect of blood glucose and plasma phosphate on phosphate clearance*

| | | Blood glucose | | |
|---|---|---|---|---|
| | | High | Normal | Low |
| Plasma phosphate | High | Maximum increase* | Increase† | Increase |
| | Normal | Increase | No change | Decrease* |
| | Low | Increase | Decrease | Maximum decrease |

* Predicted but not studied.
† Increase and decrease refer to the behavior of phosphate clearance.

depression of blood levels of glucose and phosphate results in a depression of phosphate clearance to nearly zero. This depression is prevented at low blood glucose levels if plasma phosphate is not allowed to decrease (Table V).

It would appear from the present experiments that the mechanism of fall in plasma phosphate following insulin differs from that following glucose. The small decrease in plasma phosphate after glucose administration could be explained by the observed increase in phosphate excretion. In contrast, the marked depression in plasma phosphate following insulin is associated with a striking decrease in phosphate excretion. This may be explained by increased phosphate uptake in the tissues or conversion of inorganic phosphate to other phosphate compounds (19–21).

## SUMMARY AND CONCLUSIONS

Studies on the influence of blood sugar and phosphate levels on phosphate excretion by the kidney in 40 normal men have been presented. The data indicate that the concentrations of glucose and phosphate in the blood exert powerful, sensitive and independent influences on phosphate excretion by the kidney. At hyperglycemic levels, the influence of blood glucose predominates over that of plasma phosphate, whereas at hypoglycemic levels the influence of plasma phosphate takes precedence. It is concluded that this mechanism may play an important physiologic role in the minute to minute regulation of renal phosphate excretion in man and that other factors (such as insulin) may exert their influence via alteration of blood glucose or phosphate levels.

## ACKNOWLEDGMENT

The authors wish to express their gratitude to Dr. Joseph Holmes for his many helpful suggestions.

## REFERENCES

1. Reiser, R. Phosphorus changes during the absorption of oil and glucose. J. biol. Chem. 1940, **135**, 303.
2. Howard, J. E., Hopkins, T. R., and Connor, T. B. On certain physiologic responses to intravenous injection of calcium salts into normal hyperparathyroid and hypoparathyroid persons. J. clin. Endocr. 1953, **13**, 1.
3. Briggs, A. P., Koechig, I., Doisy, E. A., and Weber, C. J. Some changes in the composition of blood due to the injection of insulin. J. biol. Chem. 1924, **58**, 721.
4. Albright, F., Bauer, W., Ropes, M., and Aub, J. C. Studies of calcium and phosphorus metabolism. IV. The effect of the parathyroid hormone. J. clin. Invest. 1929, **7**, 139.
5. Ollayos, R. W., and Winkler, A. W. Urinary excretion and serum concentration of inorganic phosphate in man. J. clin. Invest. 1943, **22**, 147.
6. Sokhey, S. S., and Allan, F. N. Relationship of phosphates to carbohydrate metabolism. I. Time relationship of changes in phosphate excretion caused by insulin and sugar. Biochem. J. 1924, **18**, 1170.
7. Levitan, B. A. Effect in normal man of hyperglycemia and glycosuria on excretion and reabsorption of phosphate. J. appl. Physiol. 1951, **4**, 224.
8. Bachmann, G., Haldi, J., Ensor, C., and Wynn, W. The effects of the injection of glucose and of fructose on the rate of excretion of urine and various constituents. Amer. J. Physiol. 1938, **124**, 77.
9. Levenson, S. M., Adams, M. A., Rosen, H., and Laskey Taylor, F. H. Studies in phosphorus metabolism in man. III. The distribution, exchange and excretion of phosphorus in man using radioactive phosphorus ($P^{32}$) as a tracer. J. clin. Invest. 1953, **32**, 497.

10. Staub, A., Springs, V., Stoll, F., and Elrick, H. A renal action of glucagon. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 57.
11. Elrick, H., Huffman, E., Hlad, C., Whipple, N., and Staub, A. Glucagon and renal function. Proc. 39th Annual Meeting of the Endocrine Society, May, 1957.
12. Bonsnes, R. W., and Taussky, H. H. On the colorimetric determination of creatinine by the Jaffe reaction. J. biol. Chem. 1945, **158**, 581.
13. Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. J. biol. Chem. 1944, **153**, 375.
14. Todd, J. C., and Sanford, A. H. Clinical diagnosis by laboratory methods; A working manual of clinical pathology, 10th ed. Philadelphia, W. B. Saunders Co., 1943, p. 101.
15. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. J. biol. Chem. 1925, **66**, 375.
16. Pitts, R. F., and Alexander, R. S. The renal reabsorptive mechanism for inorganic phosphate in normal and acidotic dogs. Amer. J. Physiol. 1944, **142**, 648.
17. Smith, P. K., Ollayos, R. W., and Winkler, A. W. Tubular reabsorption of phosphate in the dog. J. clin. Invest. 1943, **22**, 143.
18. Schiess, W. A., Ayer, J. L., Lotspeich, W. D., and Pitts, R. F. The renal regulation of acid-base balance in man. II. Factors affecting the excretion of titratable acid by the normal human subject. J. clin. Invest. 1948, **27**, 57.
19. Harrop, G. A., Jr., and Benedict, E. M. Participation of inorganic substances in carbohydrate metabolism. J. biol. Chem. 1924, **59**, 683.
20. Soskin, S., Levine, R., and Hechter, O. The relation between the phosphate changes in blood and muscle, following dextrose, insulin, and epinephrin administration. Amer. J. Physiol. 1941, **134**, 40.
21. Kaplan, N. O., and Greenberg, D. M. Radioactive phosphate as an indicator of the relationship between the phosphate changes of blood, muscle and liver, following the administration of insulin. Amer. J. Physiol. 1944, **140**, 598.

# THE ROLE OF THE THYROID GLAND IN EXPERIMENTAL TRAUMATIC SHOCK

By JACK H. OPPENHEIMER,[1] HENRY M. WISE, AND DAVID A. LASLEY[2]
WITH THE TECHNICAL ASSISTANCE OF HAROLD G. HOLMES

*(From the Biophysics Division, Directorate of Medical Research, Chemical Warfare Laboratories, Army Chemical Center, Md.)*

(Submitted for publication July 16, 1957; accepted October 31, 1957)

Comparatively little attention has been paid to the role of the thyroid gland in shock. Hamolsky, Gierlach, and Jensen (1) demonstrated that the $I^{131}$ uptake of rats subjected to tourniquet shock was depressed early in shock and that the conversion of radioiodide to the organic form was depressed late in shock. Money (2) showed that bleeding caused a depression in the four hour uptake of $I^{131}$ in rats.

Because of the meager body of information relating to this problem, it was our purpose to study several aspects of thyroid function in a traumatic shock preparation developed at the Army Chemical Center. This preparation involves the creation of uniformly lethal soft tissue wounds by the detonation of high explosive charges on the hind extremities of Texas Angora goats. Although this method of creating traumatic shock involves multiple variables including blood loss, infection, and soft tissue damage, the clinical picture in the wounded animals is surprisingly uniform and simulates conditions actually prevailing on the battlefield. Additional communications will document the physiological alterations in this preparation and will present evidence that a toxic factor, possibly related to bacterial metabolism, plays a prominent role in the demise of these animals.

In order to assess the changes in thyroidal function in shock, it was thought necessary to explore certain aspects of the normal physiology of the goat thyroid. The interrelationship of thyroid function and traumatic shock were then analyzed by study of: 1) the changes in $I^{131}$ metabolism induced by shock, and 2) the effect of thyroidal status on the survival of animals subjected to the standard injury.

[1] Present address: Department of Medicine, Duke Hospital, Durham, N. C.

[2] Present address: Department of Urology, University of Kansas School of Medicine, Kansas City, Kan.

## MATERIALS AND METHODS

*The standard preparation.* The standard wound shock preparation is described in greater detail elsewhere (3). The animals used were four to five year old castrated male Angora goats weighing between 30 and 50 kilos. Their diet consisted of hay and grain supplement with plentiful rations of salt. The animals were wounded by detonating standard tetryl (a high explosive) pellets on predetermined sites on both thighs of the animals. Hemostasis was effected within minutes after injury by the clamping of bleeding vessels. The external blood loss varied between 50 and 450 ml., but in this range the survival time of the animal bore no relation to the amount of bleeding. In this series of experiments, the mean survival time of untreated animals not subjected to experimental manipulation was 22.6 hours and the standard deviation plus or minus 4.6 hours.

*Isotopic methods.* All animals used in the isotopic studies received approximately 10 $\mu$c. carrier-free $I^{131}$, as NaI, by intravenous injection into the external jugular vein. Measurements of neck activity and that of the excised thyroid glands were made with a collimated probe (Nuclear-Chicago, Model DS-1) bearing a three-fourths inch NaI scintillation crystal. Liquid samples (2 ml.) were placed into a well-type counter (Nuclear-Chicago). All counts were recorded by a binary scaling unit (Nuclear-Chicago, Model 183). All *in vitro* counts were performed with a counting error no greater than plus or minus 5 per cent. The stability of the counting circuits was checked daily against a standard source of $Cs^{137}$. Appropriate corrections for physical decay of $I^{131}$ and for background activity were made.

*Thyroidal uptake of $I^{131}$.* In the 11 day uptake experiments, the animals were mounted on carts for a period of time sufficient for the required measurements. After the completion of the test, they were released into their stalls until the next determination. The total neck activity was determined by placing the probe 20 cm. from the geometrical center of the thyroid, as estimated by anatomical landmarks. In order to assess the nonthyroidal contribution to the total count thus determined, a curved lead shield ($\frac{3}{4} \times 6 \times 8$ inches) was placed over the thyroid and the measurement repeated. The difference in the two determinations was shown to represent a reasonable approximation of the thyroidal component of the total activity "seen" by the probe. Since it was desirable to convert serial determinations expressed in counts per minute into per cent of dose ad-

ministered, the animal was sacrificed at the end of the experiment, the thyroid excised and compared directly in activity to an aliquot of the original dose. The following relationship was then applied to convert individual readings into per cent:

$$P_t = \frac{P_{terminal}}{C_{terminal}} \times C_t,$$

where $P_t$ equals uptake at time t, expressed as per cent of dose; $P_{terminal}$ equals uptake of excised thyroid, as per cent of dose; and $C_{terminal}$ equals uptake, in counts per minute, just before sacrificing the animal and excising thyroid.

*Effect of traumatic shock on iodide metabolism.* The dynamics of iodide metabolism were studied in 10 control animals and in 11 animals subjected to the standard injury, for a period of 6 hours following the injection of a 10 $\mu$c. dose of $I^{131}$. The isotope was administered to the experimental animals within one-half hour after injury. The following determinations were made in each case: a) the 6 hour thyroidal uptake, b) the 6 hour renal excretion, and c) plasma $I^{131}$ concentration at 5, 10, 20 and 30 minutes and 1, 2, 3, 4, 5 and 6 hours after administration of the dose.

Since injured animals in the standard preparation were immobilized on carts, it was felt desirable to keep control animals in a similar position in order to insure the uniformity of experimental conditions. Other experiments in this series had shown that the glomerular filtration rate drops sharply after the animal is mounted on the cart but returns to normal values within 24 hours in the restrained prone position. For this reason, all animals employed in these experiments were placed on the carts and catheterized externally one day prior to the experiment.

At the end of the six hour period each animal was sacrificed, the thyroid excised, and the uptake directly obtained by comparing the *in vitro* count to that of an aliquot of the administered dose. This method allowed precise measurements of even small percentage uptakes of $I^{131}$.

The volume of urine excreted in the course of the experiment was measured by pooling the urine voided through an external catheter and the bladder urine aspirated at the end of the six hour period. The activity of a 2 ml. sample of this pooled urine was compared to that of a known dilution of the original dose, and the per cent of the dose excreted computed on the basis of the known volume of urine formed. The values for urinary excretion were not as reliable as those for thyroidal uptake since the loss of small quantities of urine during the manipulation frequently occurred. This introduced a systematic error into the values dependent on urinary excretion. It was felt that the error thus introduced was not sufficiently great to negate the validity of the trends observed in these experiments. The use of indwelling bladder catheters, while ideally desirable, was not technically satisfactory.

At the above designated intervals, 10 ml. samples of blood were withdrawn from an external jugular vein catheter into heparinized syringes. Two ml. plasma samples were separated from the supernatant of the centrifuged specimens, and the activity compared to that of a known dilution of the original dose. No detectable quantities of protein-bound $I^{131}$ (presumably hormonal $I^{131}$) were found in the period under consideration.

On the basis of the above determinations, the following values were computed: a) renal and thyroidal clearance of $I^{131}$, b) the volume of $I^{131}$ distribution at five minutes and six hours, and c) the mean plasma concentration.

Renal and thyroidal plasma clearance may be defined by the expression:

$$K = \frac{I_t}{\int_0^t c\,dt} \times 1{,}000,$$

where K equals renal or thyroidal clearance (ml. per minute), $I_t$ equals $I^{131}$ taken up by thyroid or excreted by kidney in time t (per cent dose), c equals instantaneous plasma concentration of $I^{131}$ (per cent dose per liter), t equals time in minutes, and 1,000 equals conversion factor from liters to ml. The function $\int_0^t c\,dt$ was obtained graphically from a curve through points representing a plot of plasma concentration as a function of time. Clearance values were normalized to body surface by applying the formula SA minus $KW^{2/3}$ where SA equals surface area ($M.^2$), W equals body weight (Kg.), and K equals 0.83, as obtained by Ritzman and Colovos (4). While clearance may well be considered a constant in normal animals in stable iodine equilibrium (5, 6), in the case of traumatized animals this function, as determined in these experiments, must be regarded as an average value over the time interval studied, since one can no longer assume stable iodine equilibrium.

The volume of $I^{131}$ distribution is obtained by dividing the dose of radioiodide retained by the body by the simultaneous plasma concentration. In the case of the five minute determination, the amount of iodide excreted in the urine and taken up in the thyroid can be neglected and the space of distribution will thus be $\frac{100}{c}$. At the end of six hours, the space can be computed by the expression $\frac{100 - (U + R)}{c}$, where c is defined above, and U and R represent the six hour thyroidal uptake and renal excretion, respectively, expressed in per cent dose. The mean plasma concentration is defined as the expression $\frac{\int_0^t c\,dt}{t}$, expressed in per cent dose per liter. The methodological considerations here employed are based upon previous analyses by Berson, Yalow, Sorrentino, and Roswit (6), Keating, Wang, Luellen, Williams, Power, and McConahey (7), and Ingbar (8).

*Protein-bound iodine (PBI) determinations.*[3] A modi-

[3] We are indebted to Lt. Colonel Thomas Jeffris, Commanding Officer, Second Army Area Medical Laboratory, Fort George Meade, Md., for allowing us the use of his facilities in the performance of the PBI determina-

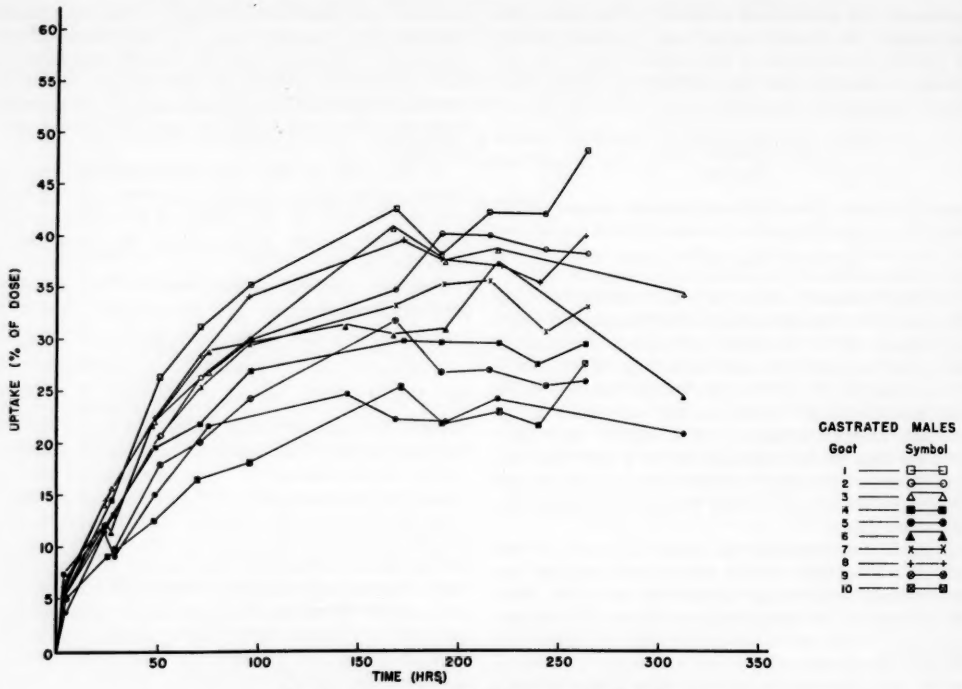



FIG. 1. THYROIDAL $I^{131}$ UPTAKE OF TEN GOATS

Goats 1 to 10 are a different set of animals from those similarly numbered in Table I.

fication of Barker's dry ashing technique (9) was employed for the determination of protein-bound stable iodine.

*Statistical methods.*[8] The "t" test was employed in all statistical treatments except in the analysis of the data contained in the section on the effect of thyroidal status on survival time. Here, the analysis of variance was employed to evaluate the statistical interrelationship of the groups involved.

## RESULTS

### *I. Some aspects of normal thyroidal metabolism of the goat*

*A. Uptake of* $I^{131}$. The results of uptakes performed on 10 castrated goats are indicated in Figure 1. Maximal uptake values varied from approximately 20 to 40 per cent of the administered dose but were not reached until about 170 hours after the injection. The nature of these curves was not related to possible metabolic derangements secondary to castration, since entirely comparable curves were plotted in five uncastrated males and five females of the same strain.

A curve representing the mean of all determinations on the 10 castrated goats is represented in the upper portion of Figure 2. In the lower half of this figure the difference between the mean maximal uptake ($U_{max}$) and the mean uptake at time t (U) was plotted as a function of t on a semilogarithmic scale. This function approximated a straight line. The straight line relationship also held true for individual animals. It has been shown (6) that the function $U_{max}$ minus U is directly related to the dose of $I^{131}$ retained in the iodide space at any instant. The straight line relationship would then imply an exponential decrease in retained $I^{131}$ over a large portion of the time interval considered.

*B. Thyroidal and renal clearance.* The mean thyroidal clearance was determined to be 2.29 ml.

tions. We also acknowledge the assistance of Mr. G. Stanley Woodson, of the Biostatistics Section, Wound Assessment Branch, Biophysics Division, Chemical Warfare Laboratories, Army Chemical Center, Md., in the statistical evaluations of our data.

per minute per M.$^2$, and the renal clearance 3.32 ml. per minute per M.$^2$ (Table I). If one assumes that radioiodide is distributed quantitatively to the thyroid and the kidney and if one discounts the small amounts of $I^{131}$ liberated as hormonal $I^{131}$, it can be shown that $U_{max} = \frac{k_u}{k_r + k_u}$, where $U_{max}$ is the maximal thyroidal uptake, $k_u$ is thyroidal clearance, and $k_r$ is renal clearance. $U_{max}$ thus calculated for the average of the 10 animals in which clearances were determined was 41 per cent. This figure may be somewhat high, since, as previously mentioned, the renal clearances may have been underestimated.

*C. Iodide space.* The iodide$^{131}$ space determined five minutes after the injection of the dose was 7.72 liters or 21 per cent body weight, and it expanded to 26.51 liters or 73 per cent body weight at the end of six hours (Table I).

*D. PBI values.* The PBI values of 73 normal goats were determined. The mean PBI was 6.06 μg. per 100 ml. serum with a standard deviation of plus or minus 1.30.

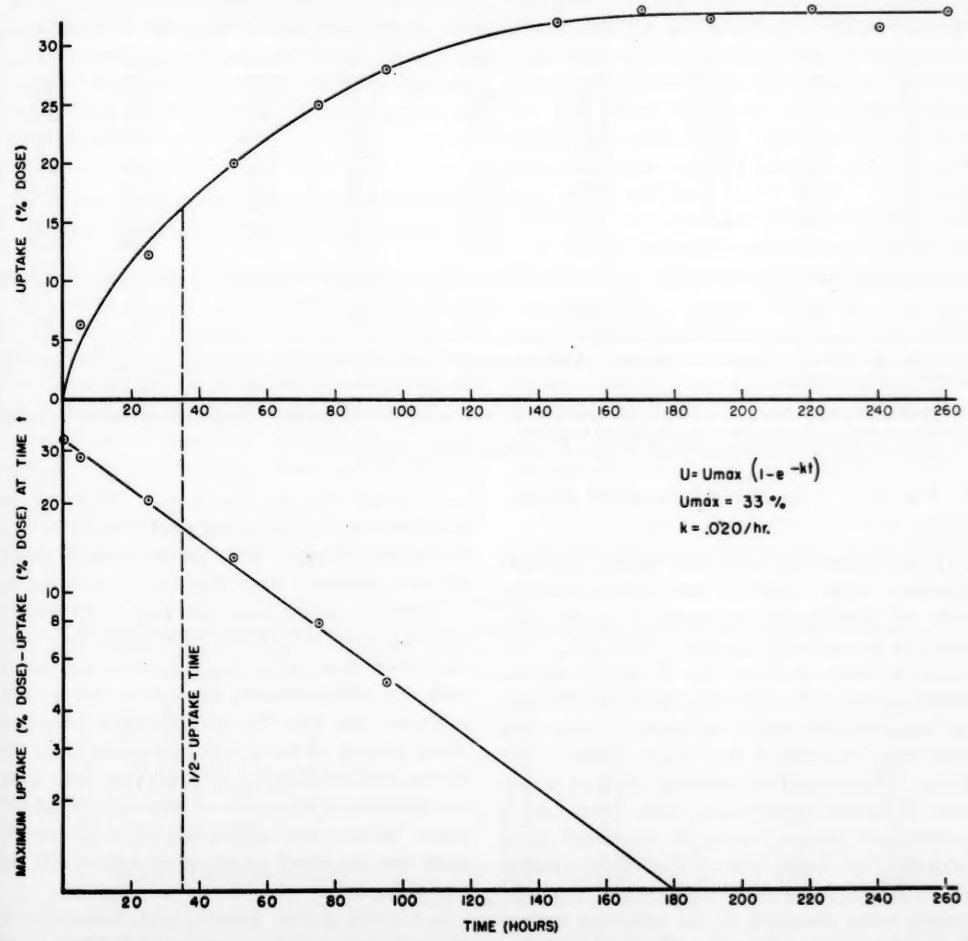



FIG. 2. THE AVERAGE OF VALUES INDICATED IN FIGURE 1 (UPPER HALF) AND $U_{max}$ MINUS U AS A FUNCTION OF TIME (SEMI-LOG PLOT, LOWER HALF)

The broken line is drawn at the time coordinate of one-half $U_{max}$.

TABLE I

*Iodide metabolism in normal and injured goats*

| | Goat no. | Weight | Average plasma concentration | Iodide space | | Thyroidal | | Renal | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 5 min. | 6 hrs. | Uptake | Clearance | Excretion | Clearance |
| | | *Kg.* | *% dose/L.* | *L.* | *L.* | *% dose* | *ml./min./M.²* | *% dose* | *ml./min./M.²* |
| Normal | 1 | 37 | 4.10 | 9.08 | 34.60 | 2.74 | 2.02 | 5.91 | 4.35 |
| | 2 | 38 | 4.81 | 8.75 | 30.37 | 1.66 | 1.02 | 1.75 | 1.08 |
| | 3 | 32 | 6.65 | 6.43 | 18.47 | 2.86 | 1.43 | 7.57 | 3.81 |
| | 4 | 35 | 6.54 | 6.16 | 20.44 | 2.85 | 1.36 | 5.97 | 2.84 |
| | 5 | 37 | 4.53 | 8.08 | 28.24 | 8.61 | 5.73 | 4.40 | 2.93 |
| | 6 | 38 | 4.96 | 9.29 | 24.56 | 4.83 | 2.89 | 5.28 | 3.16 |
| | 7 | 35 | 4.52 | 7.85 | 36.22 | 4.15 | 2.87 | 6.38 | 4.41 |
| | 8 | 35 | 5.79 | 7.10 | 22.82 | 1.76 | 0.95 | 5.59 | 3.02 |
| | 9 | 37 | 5.74 | 5.88 | 19.84 | 2.78 | 1.45 | 6.92 | 3.73 |
| | 10 | 39 | 4.28 | 8.55 | 29.49 | 4.70 | 3.20 | 5.64 | 3.84 |
| | Mean±S.D. | 36.3±2.0 | 5.19±0.93 | 7.72±1.25 | 26.51±6.23 | 3.69±2.05 | 2.29±1.46 | 5.54±1.59 | 3.32±0.97 |
| Injured | 11 | 36 | 5.94 | 6.01 | 19.80 | 1.30 | 0.67 | 6.62 | 3.40 |
| | 12* | 35 | 8.17* | 5.38 | | 1.31* | 0.64 | 3.84* | 2.14 |
| | 13 | 36 | 7.60 | 4.49 | 17.28 | 1.36 | 0.55 | 2.24 | 0.91 |
| | 14 | 37 | 7.00 | 4.68 | 22.58 | 1.10 | 0.47 | 2.03 | 0.87 |
| | 15 | 39 | 5.80 | 6.57 | 21.47 | 0.89 | 0.45 | 0.99 | 0.45 |
| | 16 | 33 | 10.01 | 3.94 | † | 1.00 | 0.33 | † | † |
| | 17 | 33 | 8.28 | 4.46 | 13.76 | 1.62 | 0.64 | 2.07 | 0.81 |
| | 18 | 35 | 6.87 | 4.91 | 20.35 | 1.29 | 0.59 | 1.05 | 0.48 |
| | 19 | 33 | 7.35 | 5.17 | 18.29 | 1.27 | 0.56 | 2.20 | 0.97 |
| | 20 | 35 | 8.06 | 5.34 | 16.42 | 1.74 | 0.68 | 4.32 | 1.68 |
| | 21‡ | 36 | 6.83‡ | 5.88 | 17.58 | 7.06‡ | 3.19‡ | 2.76‡ | 1.24 |
| | Mean±S.D. | 35.3±1.8 | 7.43±1.28 | 5.17±0.76 | 18.61±2.73 | 1.28±0.27 | 0.56±0.11 | 2.69±1.87 | 1.30±0.96 |
| | p§ | 0.3 | 0.001 | 0.001 | 0.01 | 0.01 | 0.01 | 0.01 | 0.001 |

* Animal 12 died 4 hours after injection. Values marked *, obviously related to decreased survival, are omitted from statistical analysis.

† Technical difficulty in urine collection.

‡ Animal 21 was shown to have pathological thyroid (see text). Values marked ‡, obviously related to increased thyroidal activity, are omitted from statistical analysis.

§ Significance of difference of means of control and experimental series.

### *II. The effect of traumatic shock on $I^{131}$ metabolism*

It can be seen that thyroidal uptake, thyroidal clearance, renal excretion and renal clearance were all significantly depressed in goats subjected to the standard trauma (Table I). Note should be taken of Goat No. 21 in the experimental group. The thyroidal uptake of this animal was 7.06 per cent in six hours, a value over four times as great as any other uptake in the series. Histological examination showed a picture of intense hyperplasia which fitted into a pathological category noted in occasional goats autopsied by Light (10). For these reasons values directly dependent on increased thyroidal activity were discarded in the statistical evaluation of the results. Goat No. 12 died four hours after injection of the dose. All values for this animal listed in Table II are computed on a four hour instead of a six hour basis. Those values influenced by decreased survival (thyroidal uptake, renal excretion, mean plasma concentration) are also discarded from statistical consideration.

Thyroidal uptake and clearance were apparently depressed to a greater extent from their normal values than were renal excretion and clearance. In both instances, the uptake values were depressed less than the corresponding clearance values because of the concomitant elevation of the plasma concentration of $I^{131}$ resulting both from the diminished clearances and the reduction of $I^{131}$ space. Both at five minutes and six hours the $I^{131}$ space was measured to be approximately 70 per cent of normal.

In a series of four animals, goats were allowed to concentrate maximal amounts of $I^{131}$ in their thyroid glands. One hundred ninety-two hours after the administration of the dose, two animals

were injured in the standard manner on both hind extremities, and two animals were injured on one side alone, in order to insure longer survival. Serial determinations of neck activity were performed before and after injury, the counts being converted to per cent of dose by making the appropriate comparison to the post mortem *in vitro* count, as previously described. Examination of the results shows no startling change in the thyroidal content of $I^{131}$ (Figure 3). There was some suggestion of a brief drop in Goat No. 9,487, but even this was probably within experimental error. It should be borne in mind that if the rate of turnover of hormone in the goat thyroid is at all comparable to that of the human being, only massive increases in rate of hormone discharge would have resulted in a noticeable change in our determinations.

### *III. Effect of thyroidal status on the survival of goats subjected to traumatic shock (Table II)*

In the following group of experiments, the survival times of goats in various metabolic states subjected to the standard trauma were evaluated. Groups of untreated goats were injured as simultaneous controls. Since the various control groups showed surprisingly little variation, they were consolidated for statistical analysis into a single group of 45 goats, with a mean survival time of 22.6 hours.

Ten thyroidectomized animals, wounded 10 days after the surgical procedure, showed no significant difference in survival time from control animals. Their mean PBI value before wounding was 3.26 μg. per cent, compared to the goat normal of 6.06 μg. per cent. No significant changes in weight, pulse, respiration and temperature were noted in the interval following thyroidectomy.

Thirteen animals were given 11 daily intramuscular injections of 2 mg. L-thyroxin dissolved in 1/30 N NaOH. At the end of this period the animals showed a striking increase in pulse, respiratory rate, temperature and PBI values (16.86 μg. per cent). Their mean weight loss during this period was 9.4 per cent body weight. The animals were injured 10 days after the initiation of thyroxin treatment. Survival time was decreased to 11.7 hours following injury, statistically a highly significant effect. The large standard deviation (plus or minus 8.1 hours) was due to one goat which survived for 35 hours. The NaOH vehicle had no significant effect on survival time.

A further group of survival experiments was performed to assay possible mechanisms of the thyroxin effect. The possibility that the hyperthyroid state might have induced adrenal cortical depletion presented itself. However, cortisone acetate (Merck), administered in daily 100 mg. intramuscular doses two days prior to injury and on the day of injury, did not succeed in reversing the thyroxin effect. Cortisone without thyroxin did not affect survival time.

The possibility that the thyroxin effect on survival time was mediated through weight loss was

TABLE II

*Effect of thyroidal status and related factors on survival time of animals subjected to the standard trauma*

| Experimental group | Mean S.T.* | ±S.D.† | n‡ | $P_c$§ | $P_t$‖ |
|---|---|---|---|---|---|
| A. Controls | 22.6 | 4.6 | 45 | | 0.01 |
| B. Thyroidectomized | 23.5 | 4.9 | 10 | N.S.¶ | 0.01 |
| C. Thyroxin | 11.7 | 8.1 | 13 | 0.01 | |
| D. 1/30 N NaOH (thyroxin vehicle) | 24.9 | 3.0 | 9 | N.S. | 0.01 |
| E. Thyroxin plus cortisone | 11.8 | 4.2 | 10 | 0.01 | N.S. |
| F. Cortisone | 22.7 | 4.3 | 10 | N.S. | 0.01 |
| G. Partial starvation | 18.7 | 4.9 | 10 | N.S. | 0.05 |
| H. Maximal starvation | 14.2 | 7.5 | 10 | 0.01 | N.S. |
| I. 2, 4-dinitrophenol | 16.5 | 8.7 | 10 | 0.01 | N.S. |
| J. Saline (dinitrophenol vehicle) | 21.0 | 9.9 | 9 | N.S. | 0.01 |

* Survival time.
† Standard deviation.
‡ Number of experimental animals.
§ Probability that Mean S. T. controls minus Mean S. T. experimental group equals 0.
‖ Probability that Mean S. T. thyroxin group minus Mean S. T. experimental group equals 0.
¶ Not significant ($p > 0.05$). All "p" values are less than expressed figure.

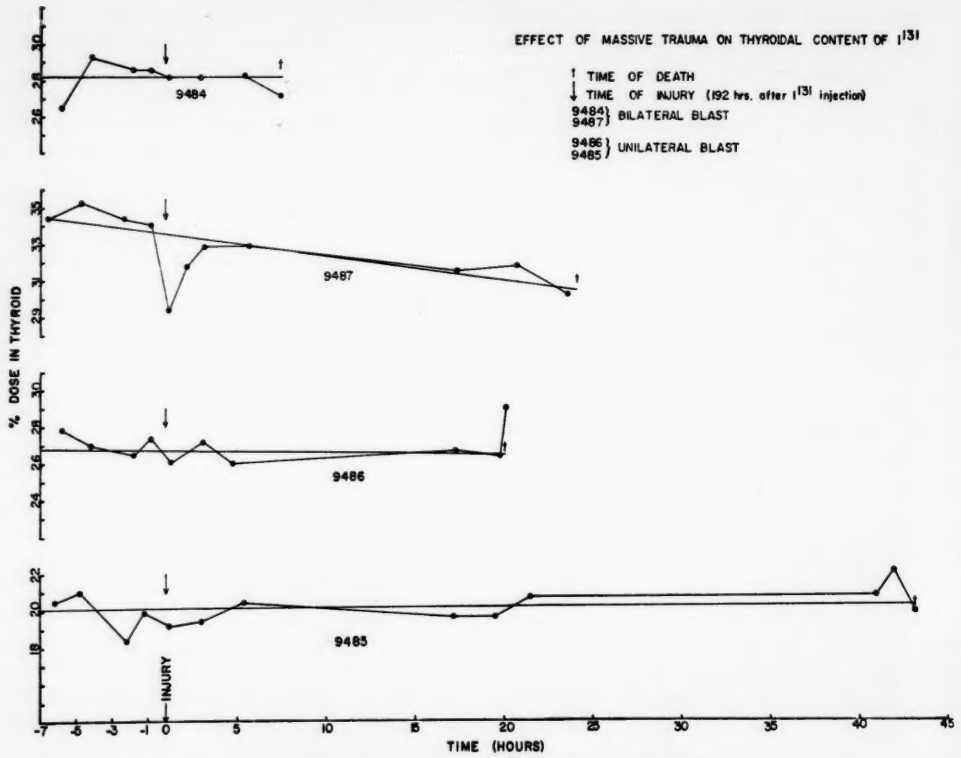



FIG. 3. EFFECT OF TRAUMA ON THYROIDAL $I^{131}$ CONTENT

examined. A group of animals were starved to produce a weight loss of 6.3 per cent body weight (the weight loss of the first nine thyroxin-treated animals). While the survival of this group was depressed (18.7 hours), it was still significantly different from the thyroxin group. Because of the difficulty of assessing the true tissue body weight in the goat, since up to 20 per cent of the measured weight may lie in the gastrointestinal system (10), animals were maximally starved for 10 days by withholding food but not water. The survival of this group (14.2 hours) did not differ significantly from the thyroxin group.

The known sensitivity of hyperthyroid animals to anoxia (11) suggested that thyroxin acted similarly in shock, where stagnant and anemic anoxia are operative. For this reason it was thought appropriate to study the effect of another compound which increased oxygen consumption. Two hundred mg. 2,4-dinitrophenol dissolved in 250 ml. saline was administered intravenously three hours prior to injury. The animals showed a markedly increased rate of respiration at the end of the three hour period. The survival of this group (16.5 hours), while not as low as the thyroxin group, did not differ from it statistically. The saline vehicle had no effect on survival time.

## DISCUSSION

The slow uptake of $I^{181}$ by the goat is a reflection of the low thyroidal (2.3 ml. per minute per $M.^2$) and renal (3.3 ml. per minute per $M.^2$) clearances when compared to corresponding values in man (10.2 ml. per minute per $M.^2$ and 20.6 ml. per minute per $M.^2$) (6). It is impossible to make any definitive conclusions on the basis of the above data regarding the comparative hormonal production of these species because of the lack of knowledge of serum level of stable iodide in the

goat. The level of PBI in the goat appeared to be quite similar to accepted values in man (12). The larger iodide space in the goat (73 per cent body weight) than in man (35 per cent body weight) (13) may possibly be attributed to the extensive gastrointestinal system of the ruminant.

It appears that the iodide concentrating capacity is severely reduced in traumatic shock. It is impossible to say on the basis of the information available whether the reduction in thyroidal clearance is a consequence of decreased blood flow or decreased iodide extraction efficiency, or a combination of the two factors. Furthermore, we cannot assume that the decreased clearance of iodide observed in these experiments reflects a decreased production of hormone, since stable iodine equilibrium conditions may not obtain. However, Harris (14) has presented evidence of decreased hormonal liberation in the rabbit subjected to various stresses, including hemorrhage. Other authors (15–17) have reported a decreased $I^{131}$ uptake in "nonspecific stress." However, it has not been ascertained to what extent this depression is due to increased renal clearance induced by cortisone or to more direct effects on the pituitary-thyroid axis by the adrenal hormones. The relationship between adrenal cortical and thyroidal metabolism has recently been reviewed by Ingbar and Freinkel (18) and Money (2).

Regarding the decreased renal clearance of $I^{131}$ in the present experiments, this would seem to accompany the decrease in effective renal blood flow and glomerular filtration previously noted in our preparation. Worthy of note is the reduction of iodide space induced by traumatic shock. It seems probable that this is related to alterations in some circulatory factor such as a decreased blood flow to the gastrointestinal tract.

The fact that the thyroidal content of $I^{131}$ did not change significantly, at least in three of our four animals, would preclude a massive discharge of labeled hormone from the gland. Since the animals were injured when the uptake curve had reached a flat maximum, the experiment could not easily demonstrate a decreased rate of discharge into the circulation. PBI determinations during shock were made in a preliminary set of experiments, and no significant changes were noted out of proportion to the decrease in protein concentration incidental to hemodilution. However, the accuracy of the method did not allow us to attach a great deal of importance to these observations.

The survival experiments would tend to indicate that the recently thyroidectomized animal can resist injury as well as the normal animal. It is certainly possible that if a longer period between thyroidectomy and injury had been allowed, the tissue changes of hypothyroidism might have influenced the survival time one way or another. Nevertheless, the results do suggest that the thyroid does not play an immediate *active* role in combating the type of stress studied.

The shortened survival of the hyperthyroid animals is consonant with the clinical observation that hyperthyroid patients present an increased operative risk. The shortened survival does not seem to result from adrenal depletion, although in the absence of information regarding adrenal cortical economy of the hyperthyroid animal in shock, it is difficult to judge whether the dosage of cortisone employed was adequate. The dinitrophenol experiments are compatible with the thesis that the thyroxin effect is related to the increased sensitivity to anoxia in a hypermetabolic animal. Weight loss as a reflection of negative nitrogen balance may be a contributory factor.

The deleterious influence of excess thyroxin combined with the fact that thyroidal clearance of iodine is sharply decreased brings to mind the speculation that traumatic shock induces a shutdown of thyroidal activity as an adaptive mechanism for the conservation of metabolic energy.

## SUMMARY

1. Traumatic shock induced a marked reduction in the thyroidal capacity to concentrate iodide, as measured by thyroidal clearance of $I^{131}$.

2. Renal clearance of $I^{131}$ was depressed, along with glomerular filtration rate and renal blood flow.

3. The space of iodide distribution was reduced by traumatic shock when measured for the first six hours following the injection of $I^{131}$.

4. No significant effect by the standard injury on the content of thyroidal $I^{131}$ was noted when the animals had accumulated maximal amounts of $I^{131}$.

5. Recently thyroidectomized animals showed no change in survival times from control values when subjected to the standard injury. Hyperthyroid goats showed marked sensitivity to traumatic shock, a phenomenon which could not be reversed by the administration of cortisone. Animals treated with 2,4-dinitrophenol showed a decrease in survival time statistically comparable to that induced by thyroxin.

6. Values for the thyroidal and renal clearance of iodide, uptake of $I^{131}$, iodide space, and level of PBI in normal goats have been ascertained.

ACKNOWLEDGMENTS

The authors wish to thank Drs. J. E. Rall and F. W. Light for their helpful comments and suggestions, and Miss Sarah Woodruff for her secretarial assistance in the preparation of this manuscript.

## REFERENCES

1. Hamolsky, M. W., Gierlach, Z. S., and Jensen, H. Uptake and conversion of radioactive iodide ($I^{131}$) by the thyroid gland *in vivo* and *in vitro* in tourniquet shock in rats. Amer. J. Physiol. 1951, **164**, 35.
2. Money, W. L. The interrelation of the thyroid and the adrenals *in* Brookhaven Symposium in Biology. Upton, New York, Brookhaven National Laboratory, 1955, No. 7, p. 137.
3. Ochsner, E. W., Jr., Jacob, S. W., and Mansberger, A. R. A new preparation for the study of experimental shock from massive wounds: I. Standardization. Surgery, In press.
4. Ritzman, E. G., and Colovos, N. F. Circular 32, University of New Hampshire, College of Agriculture, Agricultural Experimental Station, 1930.
5. Myant, N. B., Pochin, E. E., and Goldie, E. A. G. The plasma iodide clearance rate of the human thyroid. Clin. Sci. 1949, **8**, 109.
6. Berson, S. A., Yalow, R. S., Sorrentino, J., and Roswit, B. The determination of thyroidal and renal plasma $I^{131}$ clearance rates as a routine diagnostic test of thyroid dysfunction. J. clin. Invest. 1952, **31**, 141.
7. Keating, F. R., Jr., Wang, J. C., Luellen, T. J., Williams, M. M. D., Power, M. H., and McConahey, W. M. The measurement of the iodine-accumulating function of the human thyroid gland. J. clin. Invest. 1949, **28**, 217.
8. Ingbar, S. H. Simultaneous measurement of the iodide-concentrating and protein-binding capacities of the normal and hyperfunctioning human thyroid gland. J. clin. Endocr. 1955, **15**, 238.
9. Thompson, H. L., Klugerman, M. R., and Truemper, J. A method for protein-bound iodide: The kinetics and use of controls in the ashing technique. J. Lab. clin. Med. 1956, **47**, 149.
10. Light, F. Personal communication.
11. Smith, A. U., Emmens, C. W., and Parkes, A. S. Assay of thyroidal activity by a closed vessel technique. J. Endocr. 1947, **5**, 186.
12. Barker, S. B. Determination of protein-bound iodide. J. biol. Chem. 1948, **173**, 715.
13. Myant, N. B., Corbett, B. D., Honour, A. J., and Pochin, E. E. Distribution of radioiodide in man. Clin. Sci. 1950, **9**, 405.
14. Harris, G. W. The reciprocal relationship between the thyroid and adrenocortical responses to stress *in* Ciba Foundation Colloquia on Endocrinology, The Human Adrenal Cortex. Boston, Little, Brown and Co., 1955, vol. VIII, p. 531.
15. Paschkis, K. E., Cantarow, A., Eberhard, T., and Boyle, D. Thyroid function in the alarm reaction. Proc. Soc. exp. Biol. (N. Y.) 1950, **73**, 116.
16. Bogoroch, R., and Timiras, P. The response of the thyroid gland of the rat to severe stress. Endocrinology 1951, **49**, 548.
17. Van Middlesworth, L., and Berry, M. M. Iodide metabolism during anoxia, nephrectomy, trauma, avitaminoses, and starvation in the rat. Amer. J. Physiol. 1951, **167**, 576.
18. Ingbar, S. H., and Freinkel, N. ACTH, cortisone and the metabolism of iodine. Metabolism 1956, **5**, 652.

# THE CLEARANCE OF $NA^{24}$ FROM THE NORMAL AND OSTEO-ARTHRITIC KNEE JOINT AND THE RESPONSE TO INTRA-ARTERIAL PRISCOLINE® [1]

By SELVAN DAVISON AND LAWRENCE H. WISHAM

*(From the Department of Medicine [Arthritis Group], and the Department of Physical Medicine, The Mount Sinai Hospital, New York, N. Y.)*

(Submitted for publication June 17, 1957; accepted November 7, 1957)

The pathogenesis of degenerative joint disease (osteoarthritis) has been the subject of debate and conjecture for many years. Because the syndrome is found so often in older subjects, it would seem logical to correlate the general arteriosclerotic vascular changes with the degenerative joint pathology. However, the weight of investigative evidence has been against such a relationship, and the factors of "wear and tear" and "stress and strain" are considered to be the major forces in the development of degenerative joint diseases (1). In many instances where pathological changes of degenerative joint disease have been discovered, no complaint of pain or disability had been made (2, 3). It would appear that arteriosclerotic changes *per se* cannot explain degenerative joint disease (4, 5), and that vasomotor changes may play a significant role. In peripheral vascular disease, intra-arterial Priscoline® was reported to produce increased blood flow and to relieve pain and disability. It was therefore reasonable to expect a comparable effect in and around a joint and such an investigation was made by one of us (S.D.) in a series of patients with degenerative joint disease of the knee (6). Priscoline® (2-benzylimidazoline hydrochloride), an adrenolytic and sympatholytic agent with a direct action upon autonomic nerve endings, was given by the intra-arterial (femoral artery) route. In view of the beneficial subjective effect, a study was then made of intra-articular temperature changes in normal knee joints (7). This investigation demonstrated that the joint temperatures increased in normal subjects following intra-arterial sympatholytic agents (Priscoline®), presumably as a result of intra-articular vasodilatation. In another series of studies by one of us, the clearance rates of radioactive sodium from skin and muscle were investigated (8, 9). A recent study of the clearance of $Na^{24}$ from 20 normal knee joints noted 100 per cent increased clearance following local heat application, and concluded that such studies presented accurate and sensitive results in the investigation of local circulatory changes within the knee joint (10). Assuming that changes in radioactive sodium clearance from the knee joint mirror changes in the vascular bed, a study of the clearance rates of radioactive sodium from the knee joint prior to and subsequent to intra-arterial Priscoline® seemed indicated.

## MATERIAL AND METHODS

All subjects rested in the supine position for a 20 to 30 minute period prior to the beginning of the experiment. The knee joint was then entered with a 1.5 inch 20 gauge needle, and 0.1 ml. of isotonic radioactive sodium chloride solution, containing 1 to 3 microcuries of $Na^{24}$, was injected. A light weight Geiger counter was then strapped over the side of the knee opposite to the area of injection. The radioactivity present was recorded on an automatic register at one minute intervals. After six to eight minutes, 25 milligrams of Priscoline® was injected into the ipsilateral femoral artery and recording of radioactivity continued for an additional six to eight minutes. The Geiger counter remained in place throughout the experiment. The clearance half-life (the time taken for the activity initially present to be reduced to one-half) was found by plotting the data on semilogarithmic graph paper. The clearance constant (K) was calculated from the clearance half-life from the formula: K equals natural logarithm of 2 divided by half-life in minutes. In earlier reports (8, 9) we determined standard deviation based on three studies of $Na^{24}$ clearance from the same area in the same individual; two subsequent studies of the individual fell within the range of the average plus or minus twice the standard deviation. We also found that if a plus or minus 20 per cent error was arbitrarily assigned to a determination of $Na^{24}$ clearance, subsequent determinations would fall within the range so established. In the present study, we determined the significance of differences between the average clearance for normal subjects and that for all the patients, between the normals and each of the two groups

[1] This work was generously supported by a grant from the Ciba Pharmaceutical Co., Rahway, N. J.

TABLE I

*$Na^{24}$ clearance from the knee of normal subjects*

| Subject | Age (yrs.) | Clearance constant (K) minutes$^{-1}$: Prior to intra-arterial Priscoline® | Clearance constant (K) minutes$^{-1}$: After intra-arterial Priscoline® | % change |
|---|---|---|---|---|
| 1 | 42 | 0.0148 | 0.0434 | +193.2 |
| 2 | 34 | 0.0242 | 0.0442 | + 83.5 |
| 3 | 47 | 0.0131 | 0.0302 | +130.5 |
| 4 | 33 | 0.0273 | 0.0386 | + 41.4 |
| 5 | 42 | 0.0238 | 0.0366 | + 53.8 |
| 6 | 43 | 0.0110 | 0.0397 | +260.9 |
| 7 | 27 | 0.0119 | 0.0233 | + 95.8 |
| 8 | 35 | 0.0128 | 0.0447 | +249.2 |
| 9 | 28 | 0.0342 | 0.0554 | + 62.3 |
| 10 | 29 | 0.0267 | 0.0414 | + 55.1 |
| 11 | 38 | 0.0380 | 0.0231 | − 39.2 |
| 12 | 31 | 0.0254 | 0.0254 | 0 |
| 13 | 43 | 0.0192 | 0.0616 | +220.8 |
| | Average | 0.0217 | 0.0390 | |

% change of the means, 79.7

of patients, and between the two groups of patients. This was determined after finding the standard deviation of each group and comparing the observed difference to the standard error of the difference of the two groups. The formula being:

$$\sigma_\Delta = \sqrt{\frac{(\sigma_1)^2}{(n_1 - 1)} + \frac{(\sigma_2)^2}{(n_2 - 1)}},$$

where $\sigma_\Delta$ is the standard error of the difference, $\sigma$ is the standard deviation, and n is the number of observations. The chances of the differences between the groups being on a random basis were then expressed in odds.

Studies were made in 13 normal subjects between the ages of 29 and 47 years; and in 53 cases selected at random from the Arthritis Clinic with degenerative knee joint disease. The latter were between the ages of 44 and 81 years. Thirty-one of the 53 patients revealed moderate to advanced roentgenological changes typical of degenerative joint disease and 22 had normal findings or minimal X-ray changes.

In the 22 patients where distinct X-ray changes were absent, all were in the older age group and complained of pain following exercise, stiffness after resting, and local tenderness of one or both knees, with no relation to other disease or trauma. They were presumably suffering from degenerative joint disease.

## RESULTS

The clearance rates for 13 normal subjects were determined. The most rapid rate was 0.0380 minute$^{-1}$, and the slowest was 0.0110 minute$^{-1}$. The average was 0.0217 minute$^{-1}$. Eleven of the normal subjects showed a significant increase in clearance rate following intra-arterial Priscoline®; one showed a decrease, and in one instance there was no change. The increase in mean clearance following Priscoline® for this group was 79.7 per cent (see Table I).

The mean clearance constant for all 53 patients was 0.0511 minute$^{-1}$ and the odds against the difference from the mean clearance of the normal group being on a random basis were more than 500,000,000 to 1. The response to Priscoline® for all the patients showed an increase in mean clearance of 5.0 per cent.

Of the 53 patients, 31 had roentgenological changes in the knee joints. The average clearance rate for all the cases with X-ray changes was 0.0587 minute$^{-1}$. Following injection of Priscoline® 10 patients showed a significant (more than 20 per cent) increase in clearance, 11 showed a

TABLE II

*$Na^{24}$ clearance from the knee of arthritis clinic patients with X-ray evidence of osteoarthritis*

| Patient | Age (yrs.) | Clearance constant (K) minutes$^{-1}$: Prior to intra-arterial Priscoline® | Clearance constant (K) minutes$^{-1}$: After intra-arterial Priscoline® | % change |
|---|---|---|---|---|
| O. J. | 53 | 0.0990 | 0.0440 | − 55.6 |
| A. L. | 66 | 0.0408 | 0.0631 | + 54.6 |
| R. M. | 76 | 0.0257 | 0.0578 | +124.9 |
| I. F. | 71 | 0.0291 | 0.0420 | + 44.5 |
| E. M. | 65 | 0.1510 | 0.0481 | − 68.0 |
| S. M. | 71 | 0.0401 | 0.0199 | − 50.4 |
| M. E. | 73 | 0.0630 | 0.0221 | − 64.9 |
| T. F. | 69 | 0.0774 | 0.0448 | − 42.2 |
| M. C. | 65 | 0.0531 | 0.0593 | + 11.7 |
| S. T. | 61 | 0.0961 | 0.1020 | + 6.1 |
| A. S. | 61 | 0.0842 | 0.0387 | − 54.0 |
| A. G. | 65 | 0.0924 | 0.0714 | − 22.7 |
| S. G. | 60 | 0.0953 | 0.0537 | − 43.6 |
| C. M. | 65 | 0.0730 | 0.0676 | − 7.3 |
| M. B. | 67 | 0.0693 | 0.0953 | + 37.5 |
| E. B. | 81 | 0.0440 | 0.0631 | + 43.3 |
| H. Y. | 50 | 0.0774 | 0.0457 | − 41.0 |
| B. G. | 66 | 0.0194 | 0.0315 | + 62.4 |
| K. S. | 70 | 0.0540 | 0.0867 | + 60.6 |
| R. S. | 55 | 0.0346 | 0.0346 | 0.0 |
| M. T. | 65 | 0.0536 | 0.0495 | − 7.6 |
| B. H. | 65 | 0.0434 | 0.0332 | − 23.5 |
| A. A. | 64 | 0.0447 | 0.0504 | + 12.8 |
| E. K. | 80 | 0.0693 | 0.0793 | + 14.4 |
| A. B. | 75 | 0.0361 | 0.0793 | +119.7 |
| G. L. | 72 | 0.0774 | 0.0660 | − 14.7 |
| C. F. | 60 | 0.0163 | 0.0267 | + 63.8 |
| L. M. | 55 | 0.0447 | 0.0447 | 0.0 |
| P. M. | 45 | 0.0447 | 0.0321 | − 28.2 |
| D. S. | 67 | 0.0504 | 0.0566 | + 12.2 |
| A. M. | 70 | 0.0215 | 0.0385 | + 79.0 |
| Average | | 0.0587 | 0.0564 | |

% change of the means, −4.2

significant decrease, and 10 showed no significant change in clearance. The overall change in mean clearance following Priscoline® for this group was 4.2 per cent (Table II). The odds against the observed differences of the mean clearance from the normal being on a random basis were 500,000,000 to 1.

X-ray changes were absent in 22 cases and the average clearance constant for this group was 0.0404 $minute^{-1}$. The odds against the observed difference of the means as compared to the controls being random were 3,142 to 1. Ten of the patients showed a significant increase, 4 showed a significant decrease, and 8 showed no significant change in clearance rate following intra-arterial Priscoline®. The overall increase in clearance following administration of Priscoline® for this entire group was 26.2 per cent (see Table III).

In comparing the mean clearance rates for the two groups of patients we found that the odds against the observed differences being on a random basis were 99 to 1.

TABLE III

*$Na^{24}$ clearance from the knee of arthritis clinic patients without X-ray evidence of osteoarthritis*

| Patient | Age (yrs.) | Clearance constant (K) $minutes^{-1}$ Prior to intra-arterial Priscoline® | After intra-arterial Priscoline® | % change |
|---|---|---|---|---|
| S. R. | 65 | 0.0250 | 0.0536 | +114.4 |
| M. P. | 64 | 0.0231 | 0.0514 | +122.5 |
| J. B. | 44 | 0.0322 | 0.0447 | + 38.8 |
| I. K. | 77 | 0.0552 | 0.0858 | + 55.4 |
| C. I. | 72 | 0.0612 | 0.0513 | − 16.2 |
| J. V. | 67 | 0.0514 | 0.0303 | − 60.1 |
| R. Z. | 60 | 0.0236 | 0.0236 | 0.0 |
| B. L. | 56 | 0.0900 | 0.0489 | − 45.7 |
| R. T. | 60 | 0.0178 | 0.0178 | 0.0 |
| J. G. | 72 | 0.0536 | 0.0457 | − 14.7 |
| L. M. | 57 | 0.0457 | 0.0299 | − 34.6 |
| H. T. | 56 | 0.0223 | 0.0227 | + 1.8 |
| P. G. | 60 | 0.0284 | 0.0284 | 0.0 |
| D. M. | 50 | 0.0185 | 0.0618 | +234.0 |
| P. S. | 57 | 0.0631 | 0.1460 | +131.4 |
| G. S. | 54 | 0.0514 | 0.0351 | − 31.7 |
| R. L. | 68 | 0.0346 | 0.0346 | 0.0 |
| O. I. | 66 | 0.0261 | 0.0693 | +165.5 |
| M. K. | 68 | 0.0215 | 0.0447 | +108.0 |
| D. D. | 45 | 0.0326 | 0.0565 | + 73.5 |
| M. G. | 60 | 0.0361 | 0.0651 | + 80.5 |
| P. J. | 50 | 0.0770 | 0.0636 | − 17.4 |
| Average | | 0.0405 | 0.0505 | |
| | | % change of the means, 26.2 | | |

## DISCUSSION

We have divided the clinical subjects in this investigation into two main groups, those with and those without X-ray changes. In the group with X-ray changes, rapid resting clearance rates as compared to the normals were demonstrated in 77.4 per cent. Also, the average response to Priscoline® injected intra-arterially as measured by the clearance of $Na^{24}$ from the knee joint differed considerably in this group (− 4.2 per cent, as contrasted with the normals' average of + 79.7 per cent). The most logical explanation is that in joints in which osteoarthritis has been present long enough for roentgen alterations to appear, a marked degree of vasodilatation already exists and further stimulation by vasodilating agents is without effect. This hypothesis is supported by a recent study which demonstrated that the intra-articular temperature in osteoarthritis is elevated as compared to normals (11). Unpublished data of our own corroborate this report. In fact, in this group there were many studies in which the clearance rate became slower than the rate prior to the administration of Priscoline®. Since the arterioles and capillaries in the skin and muscle of the thigh and leg are in the resting state and can respond to the vasodilating drug (as evidenced by the pilomotor response, the flush and the increased skin temperature), there is an increase in blood flow to tissues other than those of the joint. This may result in a decrease of flow through the already markedly dilated vessels of the synovia subsequent to the dilatation of vessels elsewhere. We cannot account for the seven instances in which X-ray changes existed and the clearance rate was within normal range. However, as has been noted above, the odds against the average differences for the entire group as compared with the normals being on a random basis are extremely wide and indicate a high degree of probability that the differences in clearance rate are significant.

The 22 cases without X-ray change revealed a more widely scattered pattern of findings. However, when comparing the average clearance constant for this group with the average for the normal controls, statistical analysis reveals that the difference is significant. This indicates that even patients who lack X-ray changes but have symp-

toms of degenerative joint disease also have a higher degree of vasodilatation than normal.

The older age of the clinical subjects as compared to the observed normals, and, therefore, presumably the extent of arteriosclerosis present, could be considered as a factor producing the findings noted in this study. Therefore we examined for the presence of dorsalis pedis and posterior tibial pulses in all our patients. They were easily palpable except in eight instances, and a correlation could not be established between the status of the peripheral pulses and the results of our studies. If arteriosclerosis with its implied reduction in available blood flow was primarily involved, there should be an associated slower clearance of $Na^{24}$ in the resting state in the patient with degenerative joint disease and it should follow that a subject without arteriosclerosis should evince a more rapid initial $Na^{24}$ clearance. We have found the opposite to be true and this is not surprising since arteriosclerosis is not implicated in the pathogenesis of degenerative joint disease (4, 5). Consequently we do not consider arteriosclerosis to play a conspicuous role in the clearance of $Na^{24}$ from the knee joint.

Normal synovial tissue, though pale in color, contains a rich capillary bed which is not fully dilated (12). In this quiescent state, $Na^{24}$ clears slowly, but when vasodilatation is produced by the intra-arterial Priscoline®, the $Na^{24}$ clearance rate increases.

In degenerative joint disease, histological examination of the synovia reveals diffuse collagenous fibrosis, reduced vascularity and endarteritis obliterans of some of the more deeply situated arteries, but the small delicate villi contain engorged capillaries (12). This is an indication that, *in vivo*, whatever local blood supply remains in the synovia in patients with degenerative joint disease is in a state of dilatation. This is further indicated by the initial rapid clearance rates, and lack of ability of this already markedly vasodilated vascular bed to respond to further stimulation by Priscoline® intra-arterially.

That a truly inflammatory synovitis is not responsible for these results is shown by studies (12) of synovial fluid and synovia in degenerative joint disease which reveal no evidence of inflammatory changes (no leucocytic infiltration and synovial fluid cell counts well below 5,000 per cubic millimeter, and a maximum of 15 to 20 per cent polymorphonuclear cells).

We are of the opinion that our results indicate that "wear and tear" phenomena are not alone significant in the evaluation and clinical status of degenerative joint disease, but that local vascular factors play a role as well. These local vascular factors probably are of significance in the response of a joint to noxious influences. Conceivably some degree of pain in degenerative joint disease can be considered a consequence of poor or altered absorption (*i.e.*, clearance) of metabolites from the joint following exercise. Hitherto most inquiries relating to circulation and degenerative joint disease have been along purely anatomical and pathological lines. We are of the opinion that the studies herein described indicate the need for investigation into the circulatory physiology of joints.

## SUMMARY

Sixty-six studies were performed to determine the rate of clearance of radioactive sodium from the knee joint before and after the injection of Priscoline® into the ipsolateral femoral artery. In 13 normal subjects the findings indicate that in a normal joint stimulation by Priscoline® produces vasodilatation as evidenced by more rapid clearance of the $Na^{24}$.

Fifty-three patients considered to have degenerative joint disease were studied. Where distinct roentgen changes were present there was a rapid initial clearance of sodium from the knee joint in the majority of instances, as compared with the normals. There was no increase in clearance following Priscoline®.

In the group without X-ray changes, the average clearance constant was rapid as compared with the normal group. The response to Priscoline® was moderate.

## ACKNOWLEDGMENTS

The assistance of Drs. L. I. Gordon and Lena Sharney is gratefully acknowledged. The authors wish to thank Drs. William Bierman and Sergei Feitelberg for their advice and suggestions in the preparation of this paper.

## REFERENCES

1. Bennett, G. A., Waine, H., and Bauer, W. Changes in the Knee Joint at Various Ages. New York, The Commonwealth Fund, 1942.

2. Bauer, W. Studies pertaining to the origin and nature of hypertrophic arthritis. Trans. Stud. Coll. Phycns Philad. 1939, **7**, 1.
3. Amprino, R., and Bairati, A. Studi sulle trasformozioni delle cartilagini dell' uomo nell' accrescimento e nella senescenza. Z. Zellforsch. 1934, **20**, 489.
4. Heine, J. Uber die Arthritis deformans. Virchows Arch. path. Anat. 1926, **260**, 521.
5. Kling, D. H. Significance of peripheral circulatory disturbances for development of osteo-arthritis. Amer. J. med. Sci. 1939, **197**, 358.
6. Davison, S., and Wolf, M. Palliative treatment of osteoarthritis (degenerative joint disease) by the intra-arterial injection of Priscoline. N. Y. St. J. Med. 1952, **52**, 2025.
7. Davison, S., Borken, N., and Wolf, M. Elevations in temperature of joint muscle and skin following injection of Priscoline intra-arterially. J. Mt. Sinai Hosp. 1954, **21**, 98.
8. Wisham, L. H., and Yalow, R. S. Some factors affecting the clearance of $Na^{24}$ from human muscle. Amer. Heart J. 1952, **43**, 67.
9. Wisham, L. H., Yalow, R. S., and Freund, A. J. Consistency of clearance of radioactive sodium from human muscle. Amer. Heart J. 1951, **41**, 810.
10. Harris, R., and Millard, J. B. Clearance of radioactive sodium from the knee joint. Clin. Sci. 1956, **15**, 9.
11. Hollander, J. L., and Moore, R. Studies in osteoarthritis using intra-articular temperature response to injection of hydrocortisone acetate and prednisone. Ann. rheum. Dis. 1956, **15**, 4.
12. Collins, D. H. The Pathology of Articular and Spinal Diseases. Baltimore, The Williams and Wilkins Co., 1949.

# A COMPARISON OF METHODS OF MEASURING THE DIFFUSING CAPACITY OF THE LUNGS FOR CARBON MONOXIDE. INVESTIGATION BY FRACTIONAL ANALYSIS OF THE ALVEOLAR AIR

By R. MARSHALL [1]

(*From the Medical Unit, St. Bartholomew's Hospital, London, England*)

(Submitted for publication August 16, 1957; accepted November 7, 1957)

The diffusing capacity of the lungs for carbon monoxide is measured as the ml. of carbon monoxide (CO) that can be transferred from the alveoli to the blood per mm. Hg pressure difference of CO between the alveoli and the blood. This measurement can be made by steady state techniques or by the single breath method. Two steady state methods have been described recently, which differ in the methods by which the mean alveolar CO concentration is measured. In one method (1) the mean alveolar CO concentration is calculated by assuming that CO and $CO_2$ have the same dead space and that the partial pressure of $CO_2$ in the arterial blood is equal to the mean alveolar partial pressure of $CO_2$. This method requires an arterial puncture and accurate estimation of the partial pressure of $CO_2$ in the arterial blood so that its routine and repeated use in patients is limited. The other steady state method described by Bates, Boucot, and Dormer (2) uses the end tidal CO as a measure of the mean alveolar CO and, as it is a bloodless method, can more easily be used for repeated measurements on patients. These two methods give results which lie in a similar range although some of the values for normal subjects reported by Bates and Pearce (3) are low in relation to the usually accepted range. The single breath method of Krogh and Krogh (4), recently modified by Ogilvie, Forster, Blakemore, and Morton (5), is simple to carry out but comparison with the steady state method of Filley has shown (6) that the single breath method gives higher values in normal subjects; in disease the values obtained by the single breath method may be very much higher than those obtained by the steady state method. Similar results have been obtained in this laboratory when comparing the single breath method with the steady state method of Bates and co-workers (2). By this latter method patients with emphysema give a uniformly low $D_L$ [2] but many of these patients have a normal $D_L$ measured by the single breath method. Because of these findings it was decided to make further comparisons of the single breath and steady state methods of measuring $D_L$ by applying methods for fractional analysis of the alveolar air.

## METHODS

*The single breath measurements of $D_L$.* These were made using the apparatus and methods described by Ogilvie and co-workers (5). The gas mixture inspired consisted of 14 per cent helium, 0.125 per cent CO, 20 per cent $O_2$ and the remainder $N_2$. Before and after each determination on the normal subjects the alveolar air was equilibrated with the carboxyhemoglobin in the blood by breath holding for the maximum time at the functional residual capacity (F.R.C.) The patients equilibrated the blood and alveolar CO by rebreathing the expired air for 30 to 45 seconds (7). These two methods have been found to give the same result and the patients find rebreathing easier to do than breath holding. The back pressure of carbon monoxide before breath holding was calculated by correcting the equilibrated CO according to the following equation:

$$\text{Back pressure of CO in lungs during breath holding} = \text{equilibrated CO per cent} \times \frac{\text{B.H. } O_2}{\text{equil. } O_2},$$

where B.H. $O_2$ is the tension of $O_2$ in the lungs during the test, and equil. $O_2$ is the tension of $O_2$ in the rebreathing bag at the end of the equilibration. This correction is only approximate since it assumes that the hemoglobin in the capillaries is fully saturated with oxygen, but in these subjects the equilibration CO tension was small (approximately 0.1 to 0.5 per cent of the tension of CO in the alveoli during breath holding) and the error introduced is small. This back pressure of CO was sub-

[1] Cooper and Coventson Research Scholar, St. Bartholomew's Hospital. Present Address: The Nuffield Department of Surgery, The Radcliffe Infirmary, Oxford, England.

[2] The abbreviation $D_L$ is used to designate the apparent diffusing capacity of the lung for CO (5).

tracted from the expired CO per cent for calculation of $D_L$ (5).

Measurements of the single breath $D_L$ in normal subjects were made at full inspiration and at a volume approximating the F.R.C. Two measurements were made at each of the volumes. Measurements were made at full inspiration only in the patients with emphysema because of the small volume of expired air available for analysis and because, in these patients, the inspiratory capacity is small in relation to the F.R.C. The predicted normal values for $D_L$ were calculated from the regression equation of Ogilvie and co-workers (5).

*Single breath measurements of $D_L$ with fractional analysis of the expired air.* The apparatus used for the fractional analysis of the expired air is shown in Figure 1. It was essentially the same as that described by Ogilvie except that no three-way tap was inserted between the valve box and the mouthpiece, and distal to the expiratory valve a three-way tap ($T_2$) and a 4.5 meter length of rubber tubing of 2.5 cm. bore (C) connected the expiratory valve to the aspirator bottle containing the bag (A) and to the spirometer. This tube had cross wires inserted at intervals of 30 cm. in order to produce a turbulent flow of gas in the tube (8). The mouthpiece and valve assembly had a dead space of 35 ml. The procedure for fractional measurement of the diffusing capacity was similar to that described by Ogilvie (5), but the expiration after the 10 seconds breath holding was as full as possible, and immediately on its completion the tap ($T_2$) was turned to shut off the gas in the tube (C). Samples were taken from three points along the tube at known volumes from the mouthpiece by piercing the rubber tube with wide bore needles attached to well-oiled 50 ml. syringes. At each point two samples were withdrawn simultaneously. The samples were first withdrawn at the distal end of the tube. The volume along the tube at which these samples were taken was determined by the volume of gas expired. A second pair of samples was withdrawn at an intermediate point along the tube and a third pair of samples from just beyond the tap ($T_2$). The samples taken at each point of the tube were analyzed for CO and helium. The gas mixture inspired contained 14 per cent helium, 1 per cent CO, 20 per cent $O_2$ and the remainder nitrogen. The high percentage of CO was necessary because the CO samples had to be diluted to a volume large enough to completely flush the analysis tube of the infra-red CO meter. Preliminary experiments had shown that concentrations of CO in the inspired air as high as 3 per cent had no effect on $D_L$ measured by the single breath method. The syringes were calibrated to within 0.1 ml. by weighing the water that they could deliver. The CO concentration of the sample was measured by diluting 50 ml. of gas from the syringe with air in a vessel of 668 ml. capacity. After allowing an interval for mixing, the diluted gas was displaced by water and passed through the CO meter. The helium concentration was measured by passing the gas from the syringe through a small soda lime scrubber and through a katharometer.

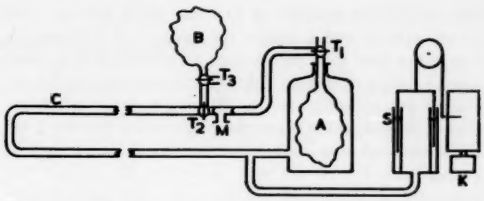



FIG. 1. DIAGRAM OF THE APPARATUS FOR MEASUREMENT OF $D_L$ WITH FRACTIONAL ANALYSIS OF THE EXPIRED AIR

A, bag containing the gas mixture to be inspired; B, bag for collecting the expired gas in the steady state; C, tube in which the expired air is held prior to sampling; M, mouthpiece; $T_1$, $T_2$, $T_3$, three-way taps; S, spirometer; K, kymograph.

Calculation of $D_L$ was made in the usual manner for each set of samples. The time that had elapsed between the beginning of the inspiration and the expiration of the sample was measured from the kymograph tracing. The lung volume used in the calculation was the same for each sample, namely the volume during breath holding. This fractional measurement of $D_L$ was carried out first in the usual way with a full inspiration from the residual volume; it was repeated with an inspiration of moderate depth (usually about 1.5 liters) taken from the F.R.C.

*Measurement of the steady state $D_L$ with fractional analysis of the alveolar air.* The apparatus used was similar to that shown in Figure 1 except that the bag (A) and aspirating bottle were replaced by a rubberized gabardine bag of 80 liters capacity containing the gas mixture to be breathed. On the side arm of the tap $T_2$ was attached another rubberized gabardine bag (B) and three-way tap ($T_3$). The taps $T_2$ and $T_3$ were turned so that at the start of the experiment the subject expired to the atmosphere through the side arm of tap $T_3$. Before the start of the experiment an equilibration sample was obtained by breath holding or rebreathing as described previously. The procedure during the experiment was as follows: The subject breathed a mixture of approximately 0.5 per cent CO in air and expired to the atmosphere through $T_3$. The patients were allowed to breathe at their normal respiratory rates but the normal subjects breathed to a metronome at 12 respirations per minute. This was done because some normal subjects, when breathing quietly, have a small tidal volume, but usually have a larger tidal volume when breathing in time to a metronome. A tidal volume of about 600 ml. is necessary to wash out the dead space so that the end tidal sample consists of alveolar air. Two and one-half minutes were allowed for a steady state to be established in the lungs. Tap $T_3$ was turned to collect the expired gas in bag B and the gas collected for a measured time, usually one and one-half minutes. During this period of collection the respirations were counted and end tidal samples of approximately 10 ml. of gas were taken from just beyond the expiratory valve during each inspiration.

The sample for analysis of CO was taken into an oiled 50 ml. syringe and a sample for analysis of $CO_2$ and $O_2$ was taken into a 20 ml. syringe lubricated with a saturated solution of lithium chloride. At the end of the period of gas collection the tap $T_3$ was turned for the patient to expire to the atmosphere and a few breaths later the kymograph was started and the tap $T_2$ was turned to connect tube C. At the next inspiration the subject was instructed to expire fully and forcibly. The patients with emphysema started their full expiration at the height of an inspiration but normal subjects started it after a normal expiration. As soon as the expiration was complete, tap $T_2$ was turned to close off the tube C and samples were taken from the tube for analysis of CO, $CO_2$ and $O_2$. The samples were analyzed for CO as described above and for $CO_2$ and $O_2$ using the Scholander gas analysis apparatus. Equilibration samples were again obtained, half a minute after the CO breathing from the normal subjects and one minute after the end of the CO breathing from the emphysema patients.

*Calculation of the mean alveolar CO by a modification of Filley's method.* The mean alveolar CO was calculated from the mixed venous $pCO_2$ of the last five patients studied. Sivertson and Fowler (9) have shown that after a period of 16 to 20 seconds of breath holding the expired air, in patients with emphysema, contains a uniform concentration of $CO_2$. This concentration is apparently the same as the mixed venous $CO_2$. In this investigation equilibration with mixed venous blood was carried out by rebreathing for 20 seconds from a bag containing 1 liter of alveolar air. This alveolar air had been obtained after a previous 20 second rebreathing period. The rebreathing was carried out immediately after the patient had completed the full expiration into the fractional analysis tube at the end of the period of steady state breathing. The assumptions which must be made in order to calculate the end capillary $pCO_2$ are the cardiac output and the pH of the blood. In this study a cardiac output of 6 liters per minute, a pH of 7.41 for the mixed venous blood, and a pH of 7.39 for the arterial blood have been assumed. The arteriovenous difference in $CO_2$ content is calculated from the measured $CO_2$ output during the steady state breathing and from the assumed cardiac output. The end capillary $pCO_2$ is calculated using the nomograph of Dill, Edwards, and Consolazio (10). Since the assumed values of cardiac output and pH affect only the arteriovenous difference of $pCO_2$, which is usually in the region of 5 or 6 mm. Hg, reasonable variations in the assumed values for cardiac output and pH will produce errors of only 1 or 2 mm. Hg in the end capillary $pCO_2$, errors which are no greater than the experimental error of direct measurement of arterial $pCO_2$.

*Fractional analysis of the expired air for helium.* This procedure was carried out using the apparatus shown in Figure 1. A 14 per cent helium mixture in air was inspired and expired *immediately* into the tube. Samples were taken at intervals along the tube. Fractional analysis of the expirate was carried out: a) after a full inspiration from the residual volume, and b) after an inspiration of about 1.5 liters from the F.R.C. Procedure b) was done in order to measure the distribution of helium in the alveolar air after a breath similar to an ordinary inspiration.

The lung volume of all subjects was measured by closed circuit spirometry and by the helium dilution method of Bates and Christie (11). The results were corrected to B.T.P.S.

TABLE I

*Physical data on five normal subjects and eleven patients with emphysema*

| Subject | Age | Sex | Height | Weight | B.S.A.* | F.R.C.† | R.V.‡ | V.C.§ |
|---|---|---|---|---|---|---|---|---|
| | *years* | | *cm.* | *Kg.* | *M.²* | *L.* | *L.* | *L.* |
| Normal | | | | | | | | |
| A. B. | 26 | M | 168 | 60.8 | 1.66 | 3.39 | 1.98 | 4.41 |
| R. W. | 28 | M | 185 | 71.0 | 1.93 | 6.72 | 3.83 | 6.52 |
| R. M. | 34 | M | 176 | 75.4 | 1.95 | 3.86 | 1.69 | 5.77 |
| M. E. | 19 | F | 161 | 47.7 | 1.47 | 3.33 | 2.08 | 3.50 |
| P. R. | 19 | F | 165 | 54.9 | 1.59 | 2.54 | 1.50 | 3.53 |
| Emphysema | | | | | | | | |
| P. F. | 53 | M | 173 | 60.2 | 1.72 | 6.61 | 5.83 | 1.95 |
| G. E. | 63 | M | 169 | 68.5 | 1.78 | 5.06 | 4.58 | 1.96 |
| T. F. | 64 | M | 178 | 63.5 | 1.79 | 3.94 | 3.43 | 2.37 |
| R. R. | 51 | M | 180 | 78.0 | 1.97 | 5.70 | 5.12 | 2.32 |
| A. G. | 63 | M | 170 | 46.2 | 1.52 | 6.12 | 5.50 | 2.39 |
| J. T. | 61 | M | 168 | 56.2 | 1.63 | 4.24 | 3.81 | 2.04 |
| H. B. | 62 | M | 165 | 69.8 | 1.77 | 3.17 | 2.69 | 2.10 |
| J. L. | 56 | M | 174 | 51.4 | 1.61 | 5.66 | 4.70 | 3.34 |
| P. C. | 53 | M | 173 | 56.8 | 1.67 | 4.73 | 3.70 | 3.20 |
| F. B. | 58 | M | 163 | 57.3 | 1.61 | 3.64 | 3.14 | 2.48 |
| S. R. | 64 | M | 163 | 59.1 | 1.62 | 3.98 | 3.45 | 1.74 |

* Body surface area.
† Functional residual capacity.
‡ Residual volume.
§ Vital capacity.

TABLE II

*Fractional analysis of helium concentration in the expired air: Expiration immediately after end of inspiration*

| Subject | Inspiration taken from | Per cent helium: Volume of sample from mouth at end of expiration (*liters*) 4.0 | 3.5 | 3.0 | 2.5 | 2.0 | 1.5 | 1.0 | 0.5 | 0.2 |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal | | | | | | | | | | |
| A. B. | R.V.* | | 11.2 | | | 10.94 | | | | 11.08 |
| | F.R.C.† | | | 3.53 | | | 3.41 | | | 3.17 |
| R. W. | R.V. | 9.36 | | | | 9.00 | | | | 8.67 |
| | F.R.C. | | | 2.98 | | | 2.78 | | | 2.63 |
| R. M. | R.V. | | 11.50 | | | 11.35 | | | | 10.67 |
| | F.R.C. | | | | 3.06 | | | 2.58 | | 2.40 |
| M. E. | R.V. | | | | 8.48 | | | 8.25 | | 8.06 |
| | F.R.C. | | | | | 4.80 | | 4.65 | | 4.35 |
| P. R. | R.V. | | | 11.58 | | | 11.35 | | | 11.33 |
| | F.R.C. | | | | 6.98 | | | 6.77 | | 6.57 |
| Emphysema | | | | | | | | | | |
| P. F. | R.V. | | | | | 9.98 | | 7.17 | | 5.73 |
| | F.R.C. | | | | | 6.56 | | 4.37 | | 3.38 |
| G. E. | R.V. | | | | | | 9.30 | 8.43 | | 6.58 |
| | F.R.C. | | | | | | 5.92 | 5.18 | | 3.72 |
| T. F. | R.V. | | | | | 10.03 | | 9.08 | | 7.82 |
| | F.R.C. | | | | | | 6.98 | 5.50 | | 3.75 |
| R. R. | R.V. | | | | | 8.48 | | 7.30 | | 6.17 |
| | F.R.C. | | | | 7.42 | | 6.40 | | | 4.57 |
| A. G. | R.V. | | | | | 9.22 | | 7.68 | | 6.23 |
| | F.R.C. | | | | | 4.28 | | 2.95 | | 2.07 |
| J. T. | R.V. | | | | | | 10.43 | 9.27 | | 8.13 |
| | F.R.C. | | | | | | | 6.62 | 5.72 | 4.73 |
| H. B. | R.V. | | | | | 8.77 | | 8.12 | | 7.28 |
| | F.R.C. | | | | | 5.91 | | 5.23 | | 4.19 |
| J. L. | R.V. | | | | 11.0 | | | 7.97 | | 6.58 |
| | F.R.C. | | | | | 5.83 | | 3.60 | | 2.50 |
| P. C. | R.V. | | | | | 9.80 | | 9.30 | | 8.48 |
| | F.R.C. | | | | | 5.53 | | 4.62 | | 4.04 |
| F. B. | R.V. | | | | | 10.60 | | 9.51 | | 7.97 |
| | F.R.C. | | | | | 6.34 | | 4.77 | | 3.58 |
| S. R. | R.V. | | | | | | 5.40 | 4.78 | | 3.36 |
| | F.R.C. | | | | | | 5.95 | 4.62 | | 2.47 |

* Residual volume.
† Functional residual capacity.

## RESULTS

The investigations were carried out on 5 normal subjects and 11 patients with emphysema. The physical data and lung volumes of these subjects are shown in Table I.

### *A. Distribution of helium in the lungs after a single breath*

The results for the normal subjects and the patients with emphysema are shown in Table II. These experiments were carried out to investigate

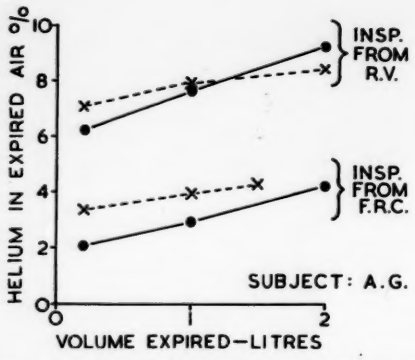



FIG. 2. DISTRIBUTION OF HELIUM IN THE EXPIRED AIR AFTER A SINGLE BREATH FROM THE RESIDUAL VOLUME (R.V.) OR FROM THE FUNCTIONAL RESIDUAL CAPACITY (F.R.C.) (PATIENT WITH EMPHYSEMA)

●, expiration immediately after the inspiration; ×, expiration after breath holding for 10 seconds.

the gas distribution in the lungs of the subjects and to compare the distribution after large breaths from the residual volume (as in the single breath test) and inspiration of a moderate sized breath from the F.R.C. (as in the steady state tests).

TABLE III

*Measurement of $D_L$ by the single breath method*

| Subject | Predicted $D_L$ | Lung volume | $D_L$ |
|---|---|---|---|
| | *ml./min./mm. Hg* | *liters B.T.P.S.* | *ml./min./mm. Hg* |
| Normal | | | |
| A. B. | 24.5 | 7.31 | 40.7 |
| | | 4.46 | 30.8 |
| R. W. | 29.6 | 9.70 | 48.6 |
| | | 7.00 | 36.6 |
| R. M. | 30.0 | 7.41 | 31.1 |
| | | 4.50 | 24.7 |
| M. E. | 20.9 | 5.05 | 25.2 |
| | | 3.59 | 21.9 |
| P. R. | 23.0 | 4.99 | 26.6 |
| | | 2.99 | 23.3 |
| Emphysema | | | |
| P. F. | 25.6 | 8.13 | 9.0 |
| G. E. | 26.8 | 7.23 | 25.5 |
| T. F. | 27.0 | 5.90 | 8.3 |
| R. R. | 30.2 | 7.92 | 25.0 |
| A. G. | 21.9 | 8.79 | 18.1 |
| J. T. | 23.9 | 6.04 | 23.6 |
| H. B. | 27.9 | 6.04 | 20.0 |
| J. L. | 23.5 | 7.82 | 10.7 |
| P. C. | 24.7 | 7.38 | 19.8 |
| F. B. | 23.5 | 5.94 | 18.8 |
| S. R. | 23.7 | 5.16 | 21.7 |

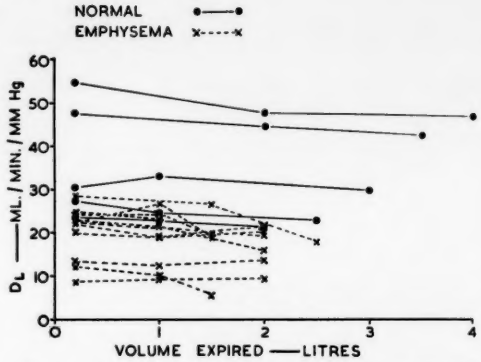



FIG. 3. VARIATIONS IN $D_L$ IN DIFFERENT FRACTIONS OF THE EXPIRED AIR AFTER BREATH HOLDING FOR 10 SECONDS

The effect of 10 seconds breath holding on the distribution of inspired gas was also investigated. The normal subjects had an almost even distribution of gas with both types of inspiration. The patient with emphysema showed uneven distribution of the inspired helium with both types of inspiration. Breath holding for 10 seconds reduced, but did not abolish, the uneven gas distribution (Figure 2).

### *B. Single breath $D_L$ measurements*

The results of these measurements are shown in Table III. Six of the eleven patients with

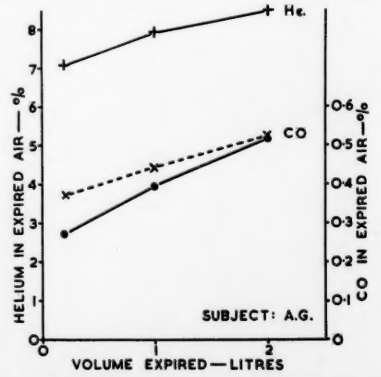



FIG. 4. THE CONCENTRATIONS OF CO AND HELIUM IN THE DIFFERENT FRACTIONS OF THE EXPIRED AIR OF A PATIENT WITH EMPHYSEMA AFTER BREATH HOLDING FOR 10 SECONDS

●, CO concentration measured in the expired air; ×, CO concentration corrected for the time required for expiration.

emphysema had a single breath $D_L$ within one standard error of the normal value predicted by the formula of Ogilvie and associates (5).

### *C. Fractional measurement of $D_L$ by the single breath method*

The results obtained in these experiments are shown in Table IV and in Figures 3 and 4. There was no appreciable difference in the results obtained with breath holding after a full inspiration from the residual volume and those obtained with an inspiration from the F.R.C. of about 1.5 liters. The $D_L$ of the fractional samples of the alveolar gas of the normal subjects shows a small rise in the later expired samples in some of the subjects but in most $D_L$ is approximately uniform throughout expiration.[3] Similar results were obtained

[3] The later expired samples contain less $O_2$ and this will increase the $D_L$ of these samples, but the effect is small. For example, if the early expired sample contains 17 per cent $O_2$ ($\theta = 0.71$) and the later expired one 14 per cent $O_2$ ($\theta = 0.77$) (12), and if $D_M = 60$ ml./

TABLE IV

*Single breath $D_L$ with fractional analysis of the expired air*

| Subject | Lung volume during breath holding | Total volume expired | $D_L$ Volume of sample from mouth at end of expiration (*liters*) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 4.0 | 3.5 | 3.0 | 2.5 | 2.0 | 1.5 | 1.0 | 0.2 |
| | *liters* | *liters* | | | | | | | | |
| Normal | | | | | | | | | | |
| A. B. | 7.22 | 4.50 | | 42.6 | | | 44.6 | | | 47.7 |
| | 5.62 | 2.92 | | | | | 36.9 | | 36.6 | 39.2 |
| R. W. | 9.70 | 5.21 | 47.2 | | | | 47.8 | | | 54.8 |
| | 8.29 | 3.94 | | | 37.5 | | | 44.3 | | 44.5 |
| R. M. | 7.06 | 4.46 | | | 29.8 | | | | 33.1 | 30.5 |
| | 5.60 | 3.33 | | | | 26.5 | | | 26.5 | 25.8 |
| M. E. | 4.85 | 2.50 | | | | | 21.3 | | 22.9 | 23.6 |
| | 4.85 | 2.49 | | | | | 21.0 | | 22.8 | 23.3 |
| P. R. | 5.00 | 3.12 | | | | 22.8 | | | 24.5 | 27.4 |
| | 4.83 | 2.96 | | | | | 20.8 | | 21.3 | 25.7 |
| Emphysema | | | | | | | | | | |
| P. F. | 7.99 | 1.79 | | | | | | 5.8 | 10.0 | 12.1 |
| | 7.99 | 1.73 | | | | | | 0.7 | 8.8 | 12.9 |
| G. E. | 7.26 | 1.88 | | | | | | 19.6 | 24.4 | 24.4 |
| | 7.26 | 1.92 | | | | | | 15.0 | 23.4 | 24.2 |
| T. F. | 6.43 | 2.50 | | | | | 9.4 | | 9.6 | 8.7 |
| | 6.40 | 2.46 | | | | | 7.0 | | 9.3 | 9.5 |
| R. R. | 8.95 | 2.90 | | | | 18.0 | | 26.8 | | 28.9 |
| | 8.40 | 2.87 | | | | 19.3 | | 31.1 | | 25.5 |
| A. G. | 8.82 | 2.46 | | | | | 16.0 | | 21.5 | 23.0 |
| | 8.70 | 2.31 | | | | | | 12.0 | 16.9 | 19.1 |
| J. T. | 6.28 | 1.75 | | | | | | 19.2 | 26.8 | 22.5 |
| | 6.26 | 1.84 | | | | | | 18.6 | 26.2 | 26.3 |
| H. B. | 6.44 | 2.33 | | | | | | | 22.9 | 24.4 |
| | 6.35 | 2.41 | | | | | 14.5 | | 21.8 | 22.6 |
| J. L. | 8.10 | 2.71 | | | | | 13.6 | | 12.5 | 13.5 |
| | 7.74 | 2.95 | | | | 12.9 | | 11.1 | | 11.5 |
| P. C. | 7.06 | 2.86 | | | | | 19.5 | | 21.0 | 22.4 |
| | 6.59 | 2.44 | | | | | 16.3 | | 18.1 | 18.5 |
| F. B. | 6.03 | 2.44 | | | | | 20.1 | | 19.0 | 22.8 |
| | 5.99 | 2.40 | | | | | 17.5 | | 19.5 | 22.9 |
| S. R. | 5.25 | 2.20 | | | | | | 21.5 | 24.1 | 23.3 |
| | 5.25 | 1.86 | | | | | | 14.8 | 18.4 | 17.6 |

TABLE V

*The steady state $D_L$ with fractional analysis of a full expiration: Normal subjects*

| Subject | Minute volume (L.) | Resp. per min. | End tidal gas (%) | | | Mixed expired gas (%) | | | Vol. of sample from mouth (L.) | Fractional sample | | $D_L$ calculated from | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $CO_2$ | $O_2$ | CO | $CO_2$ | $O_2$ | CO | | CO | CO corrected for time | End tidal | 0.2 L. sample |
| A. B. | 11.0 | 12 | 4.77 | 16.56 | 0.1861 | 3.42 | 17.84 | 0.2769 | 3.0 | 0.2218 | 0.2043 | 27.4 | 22.7 |
| | | | | | | | | | 2.0 | 0.2039 | 0.2172 | | |
| | | | | | | | | | 1.0 | 0.1883 | 0.2160 | | |
| | | | | | | | | | 0.2 | 0.1736 | 0.2220 | | |
| R. W. | 10.9 | 12 | 5.39 | 15.12 | 0.1011 | 3.48 | 17.51 | 0.1995 | 2.5 | 0.0703 | 0.0727 | 36.9 | 55.5 |
| | | | | | | | | | 1.5 | 0.0705 | 0.0744 | | |
| | | | | | | | | | 1.0 | 0.0667 | 0.0715 | | |
| | | | | | | | | | 0.2 | 0.0606 | 0.0672 | | |
| R. M. | 9.9 | 12 | 5.47 | 15.32 | 0.1212 | 3.65 | 17.02 | 0.2075 | 3.0 | 0.1214 | 0.1106 | 22.9 | 26.6 |
| | | | | | | | | | 2.5 | 0.1084 | 0.1069 | | |
| | | | | | | | | | 2.0 | 0.1072 | 0.1085 | | |
| | | | | | | | | | 1.0 | 0.1045 | 0.1087 | | |
| | | | | | | | | | 0.2 | 0.0976 | 0.1064 | | |
| M. E. | 9.3 | 12 | 4.71 | 16.95 | 0.1783 | 3.20 | 18.34 | 0.2700 | 2.0 | 0.2030 | 0.1766 | 21.4 | 21.6 |
| | | | | | | | | | 1.5 | 0.1800 | 0.1724 | | |
| | | | | | | | | | 1.0 | 0.1713 | 0.1745 | | |
| | | | | | | | | | 0.5 | 0.1628 | 0.1714 | | |
| | | | | | | | | | 0.2 | 0.1630 | 0.1746 | | |
| P. R. | 7.6 | 12 | 5.10 | 16.20 | 0.1358 | 3.79 | 17.52 | 0.1983 | 1.5 | 0.1278 | 0.1147 | 19.0 | 24.4 |
| | | | | | | | | | 1.0 | 0.1084 | 0.1102 | | |
| | | | | | | | | | 0.5 | 0.1020 | 0.1090 | | |
| | | | | | | | | | 0.2 | 0.0942 | 0.1076 | | |

for the patients with emphysema although in some of the patients the $D_L$ of the first expired sample was low. These low values were due to admixture of the sample with dead space gas and occurred when the sample was taken within a few hundred ml. of the total volume expired (see Table IV). If the early samples are excluded the patients with emphysema are seen to have a uniform $D_L$ in the different fractions of the expired air. In these patients the time required for expiration of the last sample may be about one-third of the total breath holding time. During expiration the intrathoracic pressure is high, but Ogilvie and coworkers (5) have shown that a positive intrathoracic pressure has only a small effect on $D_L$. Thus, there is no evidence of large changes of diffusing capacity in different regions of the lungs of these patients, but unfortunately, owing to admixture with the dead space gas, this method is not precise enough to detect a low $D_L$ in the regions from which the first few hundred ml. of expired gas originate. The word "region" is used here to denote the site of origin of the gas which is expired at any particular time of expiration and does not necessarily denote an anatomical division of the lungs.

minute/mm. Hg and $V_e = 60$ ml., the $D_L$ of the early sample is 24.9 ml./minute/mm. Hg, and of the later sample 26.1 ml./minute/mm. Hg.

### *D. The steady state $D_L$ with fractional analysis of the alveolar air*

The results obtained in these experiments are shown in Tables V and VI. The concentration of CO in the expired air of the subjects was corrected for the time required for expiration, assuming that CO was taken up at the rate denoted by the single breath $D_L$ measured with the breath held at the F.R.C. (see Appendix). The relationship between the corrected CO concentration and the expired volume of a normal subject is shown in Figure 5. It can be seen that the concentration of CO is uniform throughout the lung. The end tidal sample is rather high in relation to the fractional alveolar samples in four of the five normal subjects despite the moderately large tidal volumes and the use of a valve assembly with a small dead space (35 ml.). This difference is probably produced by the subject taking a larger

TABLE VI

TABLE VI

*The steady state $D_L$ with fractional analysis of a full expiration: Patients with emphysema*

| Subject | Minute volume | Resp. per min. | CO in inspired gas | Mixed expired gas (%) | | | End tidal gas (%) | | | Fractional sample | | | | | Mean alveolar CO (Filley's) method | $D_L$ calculated from | | | Single breath $D_L$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Vol. of sample from mouth | | | | CO corrected for time of exp. | | End tidal | 0.2 L. sample | Mean alveolar CO | |
| | | | | $CO_2$ | $O_2$ | CO | $CO_2$ | $O_2$ | CO | | $CO_2$ | $O_2$ | CO | | | | | | |
| | *L.* | | % | | | | | | | *L.* | % | % | % | % | % | | | | |
| P. F. | 13.1 | 22 | 0.3765 | 2.25 | 18.54 | 0.2753 | 4.04 | 16.66 | 0.2301 | 1.5 | | | 0.2100 | 0.2050 | | 7.4 | 10.4 | | 9.0 |
| | | | | | | | | | | 1.0 | | | 0.1876 | 0.1904 | | | | | |
| | | | | | | | | | | 0.5 | | | 0.1638 | 0.1769 | | | | | |
| | | | | | | | | | | 0.2 | | | 0.1442 | 0.1649 | | | | | |
| G. E. | 13.1 | 17 | 0.4930 | 2.31 | 18.65 | 0.3620 | 4.31 | 16.70 | 0.2980 | 1.5 | | | 0.3703 | 0.3452 | | 7.6 | 11.0 | | 25.5 |
| | | | | | | | | | | 1.0 | | | 0.3330 | 0.3170 | | | | | |
| | | | | | | | | | | 0.5 | | | 0.2665 | 0.2665 | | | | | |
| | | | | | | | | | | 0.2 | | | 0.1961 | 0.2065 | | | | | |
| T. F. | 17.9 | 34 | 0.4615 | 1.59 | 19.35 | 0.3780 | 2.64 | 18.01 | 0.3500 | 1.5 | 2.08 | 18.62 | 0.3539 | 0.3461 | | 5.6 | 7.4 | | 8.3 |
| | | | | | | | | | | 1.0 | 3.02 | 17.57 | 0.2910 | 0.2831 | | | | | |
| | | | | | | | | | | 0.5 | 3.59 | 16.71 | 0.2610 | 0.2763 | | | | | |
| | | | | | | | | | | 0.2 | 3.99 | 16.01 | 0.2295 | 0.2640 | | | | | |
| R. R. | 11.2 | 19 | 0.5230 | 2.43 | 18.32 | 0.3647 | 4.08 | 16.41 | 0.2808 | 1.5 | 2.40 | 18.31 | 0.3575 | 0.3292 | | 8.4 | 16.2 | | 25.0 |
| | | | | | | | | | | 1.0 | 4.06 | 16.36 | 0.2429 | 0.2355 | | | | | |
| | | | | | | | | | | 0.5 | 5.00 | 14.92 | 0.1712 | 0.1846 | | | | | |
| | | | | | | | | | | 0.2 | 5.22 | 13.69 | 0.1174 | 0.1499 | | | | | |
| A. G. | 10.6 | 26 | 0.4632 | 2.46 | 18.54 | 0.3100 | 4.86 | 16.27 | 0.2421 | 1.0 | 3.58 | 17.38 | 0.2728 | 0.2696 | | 9.0 | 13.0 | | 18.1 |
| | | | | | | | | | | 0.5 | 5.94 | 14.86 | 0.1739 | 0.1906 | | | | | |
| | | | | | | | | | | 0.2 | 6.39 | 13.95 | 0.1341 | 0.1710 | | | | | |
| J. T. | 9.4 | 19 | 0.4420 | 2.33 | 18.36 | 0.3035 | 4.50 | 16.09 | 0.2142 | 1.0 | 3.31 | 17.28 | 0.2490 | 0.2200 | | 7.9 | 17.4 | | 23.6 |
| | | | | | | | | | | 0.5 | 5.68 | 14.54 | 0.1279 | 0.1225 | | | | | |
| | | | | | | | | | | 0.2 | 6.13 | 13.45 | 0.0876 | 0.0996 | | | | | |
| H. B. | 11.0 | 19 | 0.5380 | 2.76 | 18.22 | 0.3365 | 4.37 | 16.62 | 0.2650 | 1.5 | 2.56 | 18.43 | 0.3600 | 0.3168 | 0.1440 | 10.8 | 16.1 | 19.0 | 20.0 |
| | | | | | | | | | | 1.0 | 3.81 | 17.04 | 0.2820 | 0.2586 | | | | | |
| | | | | | | | | | | 0.5 | 4.55 | 16.05 | 0.2183 | 0.2290 | | | | | |
| | | | | | | | | | | 0.2 | 4.97 | 15.04 | 0.1290 | 0.1817 | | | | | |
| J. L. | 10.4 | 20 | 0.5590 | 1.92 | 18.92 | 0.4260 | 3.46 | 16.98 | 0.3391 | 1.5 | 3.64 | 16.64 | 0.2960 | 0.2856 | 0.2080 | 5.4 | 8.3 | 8.2 | 10.7 |
| | | | | | | | | | | 1.0 | 4.29 | 15.78 | 0.2444 | 0.2410 | | | | | |
| | | | | | | | | | | 0.2 | 4.59 | 14.74 | 0.2050 | 0.2208 | | | | | |
| P. C. | 9.33 | 15 | 0.5085 | 2.62 | 18.08 | 0.3246 | 4.36 | 16.05 | 0.2479 | 1.5 | 3.69 | 16.84 | 0.2516 | 0.2460 | 0.1278 | 9.1 | 15.1 | 16.3 | 19.8 |
| | | | | | | | | | | 1.0 | 4.63 | 15.63 | 0.1904 | 0.1938 | | | | | |
| | | | | | | | | | | 0.5 | 4.86 | 15.03 | 0.1595 | 0.1765 | | | | | |
| | | | | | | | | | | 0.2 | 5.04 | 14.10 | 0.1212 | 0.1526 | | | | | |
| F. B. | 14.3 | 28 | 0.4765 | 2.21 | 18.97 | 0.3487 | 3.60 | 17.72 | 0.2700 | 1.5 | 2.66 | 18.72 | 0.3295 | 0.3124 | 0.1583 | 9.0 | 10.7 | 14.0 | 18.8 |
| | | | | | | | | | | 1.0 | 3.59 | 17.84 | 0.2689 | 0.2640 | | | | | |
| | | | | | | | | | | 0.5 | 4.11 | 17.17 | 0.2195 | 0.2440 | | | | | |
| | | | | | | | | | | 0.2 | 4.48 | 16.48 | 0.1676 | 0.2290 | | | | | |
| S. R. | 8.3 | 18 | 0.5150 | 2.34 | 18.22 | 0.3472 | 4.45 | 15.77 | 0.2662 | 1.0 | 3.59 | 16.81 | 0.2849 | 0.2584 | 0.0449 | 6.7 | 15.6 | 25.1 | 21.7 |
| | | | | | | | | | | 0.5 | 5.52 | 13.86 | 0.1618 | 0.1618 | | | | | |
| | | | | | | | | | | 0.2 | 6.39 | 11.52 | 0.0836 | 0.1179 | | | | | |

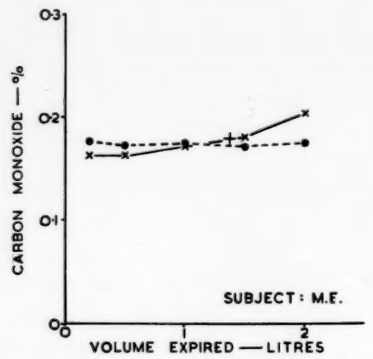



FIG. 5. CONCENTRATION OF CO IN FRACTIONS OF THE EXPIRED AIR OF A NORMAL SUBJECT IN THE STEADY STATE

×, CO concentration measured in the expired air; ●, CO concentration corrected for the time required for expiration; +, end tidal sample.

breath before the final full expiration. The $D_L$ calculated from the end tidal sample is therefore lower in most subjects than that calculated from the last expired sample, although in only one subject is this difference large.

The patients with emphysema do not have a uniform concentration of CO in the alveolar air (Figure 6), even when this is corrected for time. Figure 6 shows the fractional analysis for CO and $CO_2$ of the expired air of Patient H.B. when this patient was in the steady state. Since the alveolar CO is not uniform, the end tidal CO concentration bears no relationship to the mean alveolar CO. The $D_L$ calculated, using the different fractional samples as measures of alveolar CO, will vary widely. The values obtained for $D_L$ using a modification of Filley's method for calculating the mean alveolar CO are shown also in Table VI.

In the last five patients studied the ventilation-perfusion ratios ($\dot{V}/Q$) have been calculated by the method of Rahn and Fenn (13) for each of the samples taken during the full expiration at the end of the steady state period. These values are shown in Table VII in the form of ratios indicating the variation of the $\dot{V}/Q$ of the 1.0 liter sample to that of the last expired sample at 0.2 liter. In this table is shown also the distribution of helium in the lungs during a single breath inspired from the F.R.C. and from the residual volume. The relationship of $\dot{V}/Q$ of the 1.0 liter sample to that of the 0.2 liter sample has been corrected for the extra time required for expiration of the 0.2 liter sample. This correction has been made on the assumption that, whereas the ventilation and perfusion of the 1.0 liter sample both occupied 60 seconds and the ventilation of the 0.2 liter sample occupied 60 seconds, the perfusion of this sample occupied 60 plus t seconds, where t is the time interval between the expiration of the 1.0 and 0.2 liter samples. The ventilation ratio of the 1.0 and 0.2 liter samples, determined by the helium concentration of the samples after a single breath of helium, is usually less when the breath is taken from the residual volume than when the breath is taken from the F.R.C. Both of these values are shown in Table VII. Some of the values in this table have been omitted when the inspiration was taken from well above the F.R.C. The ratio of the $\dot{V}/Q$ of the 1.0 liter sample to that of the 0.2 liter sample is of the same order as the ratio of the ventilation of these samples, and suggests that the perfusion per unit volume of the lung from which the two samples originate is the same.

## DISCUSSION

The different values obtained for the $D_L$ of normal subjects by the single breath method and by the steady state method could be due to a number of factors. Forster, Fowler, and Bates (14) have pointed out that steady state methods

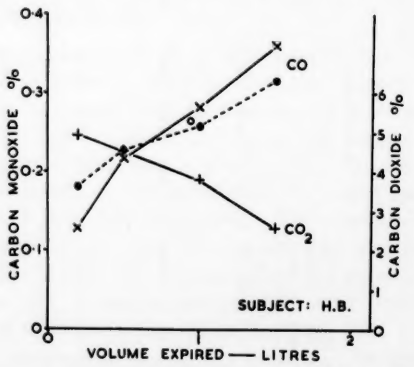



FIG. 6. CONCENTRATIONS OF CO AND $CO_2$ IN FRACTIONS OF THE EXPIRED AIR OF A PATIENT WITH EMPHYSEMA IN THE STEADY STATE

×, CO concentration measured in the expired air; ●, CO concentration corrected for the time required for expiration; ○, end tidal sample of CO.

TABLE VII

*Comparison between ventilation and ventilation-perfusion ratios in five patients with emphysema*

| Patient | $\frac{\dot{V}/Q \text{ 1.0 L. sample}}{\dot{V}/Q \text{ 0.2 L. sample}}$ | Interval between exp. of 1.0 and 0.2 L. samples (sec.) (= t) | $\frac{\dot{V}/Q \text{ 1.0 L.}}{\dot{V}/Q \text{ 0.2 L.}} \times \frac{60}{(60+t)}$ | $\frac{\text{He \%, 1.0 L. sample}}{\text{He \%, 0.2 L. sample}}$ | |
|---|---|---|---|---|---|
| | | | | Insp. from R.V.* | Insp. from F.R.C.† |
| H. B. | 1.36 | 6.26 | 1.23 | 1.13 | 1.25 |
| J. L. | 1.20 | 3.44 | 1.13 | 1.21 | ‡ |
| P. C. | 1.31 | 4.66 | 1.22 | 1.10 | 1.25 |
| F. B. | 1.31 | 5.19 | 1.21 | 1.19 | 1.33 |
| S. R. | 1.44 | 6.34 | 1.30 | 1.42 | ‡ |

* Residual volume.
† Functional residual capacity.
‡ These patients started the inspiration from a level considerably above the F.R.C.

may give inaccurate results if there is a disproportion between ventilation and diffusion of different parts of the lung. In normal subjects this and previous investigations (15) have shown that the ventilation is approximately uniform after a single breath of a foreign gas. The $D_L$ has also been found to be uniform throughout the expired air; thus, in normal subjects unevenness of ventilation and diffusion cannot be the cause of the difference in results by the two methods and further examination of the methods is necessary.

*The steady state method using end tidal samples as a measure of the mean alveolar concentration of CO ($F_{\bar{A}co}$)*

In this method $D_L$ is calculated by the following equation:

$$D_L = \frac{\text{uptake of CO (ml. per minute)}}{F_{\bar{A}co} \times (B - 47)}.$$

The uptake of CO can be measured accurately as the difference between the CO inspired and that expired. $F_{\bar{A}co}$ can be measured in normal subjects by the use of end tidal samples (Figure 5) provided that the tidal volume is 600 ml. or more, so that complete washout of the dead space can take place, and the instrumental dead space is small. Previous experience with a valve assembly having a dead space of 65 ml., which was of irregular shape and therefore not easily flushed out, showed that with this valve assembly true end tidal samples may not be obtained even with a tidal volume of 1 liter. The concentration of $CO_2$ in the alveolar gas at the end of a normal expiration lies between the maximum and minimum concentrations which are reached during the respiratory cycle (16). The end tidal sample of 60 will also lie between the maximum and minimum levels, rather closer to the minimum level, so that the value of $F_{Aco}$ will be too low and the resultant $D_L$ will tend to be too high.

*The single breath method*

The equation on which this method is based is as follows:

$$D_L = \frac{\text{ln.} \frac{F_{INco}}{F_{Eco}} \times V_A \times 60}{t \times (B - 47)}, \qquad (5)$$

where $F_{INco}$ is the initial concentration of CO in the lungs, $F_{Eco}$ is the concentration expired after time t seconds, $V_A$ (ml. S.T.P.D.) is the alveolar volume, and B is the barometric pressure in mm. Hg.

The term $\frac{\text{ln.} \frac{F_{INco}}{F_{Eco}}}{t}$ is the slope of alveolar concentration of CO plotted against time and in these experiments has been measured over the interval of 0 to 10 seconds. Forster, Fowler, Bates, and Van Lingen (17) have reported that this initial slope may be two to five times greater than between 40 and 60 seconds when the breath is held. This non-linearity of the logarithmic decay curve has been ascribed by Forster and co-workers (17) to variations in the diffusing capacity per unit gas volume throughout the lung. Since, in normal subjects, the ventilation and the $D_L$ are approximately uniform in different fractions of the expired air, it is surprising if marked differences in diffusing capacities per unit lung volume exist. The experiment of breath holding for different

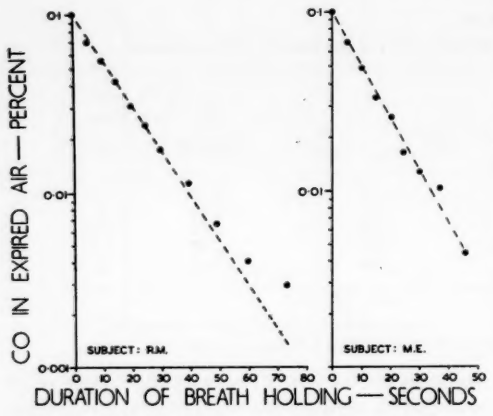



FIG. 7. THE DISAPPEARANCE OF CO FROM THE LUNGS OF NORMAL SUBJECTS WHO ARE BREATH HOLDING AT THE FULL INSPIRATORY POSITION

times has been repeated in Subjects R.M. and M.E. with the results shown in Figure 7. The breath was held at full inspiration and correction has been made at each point for the back pressure of CO existing in the blood. In Subject M.E. the CO concentration falls exponentially with time up to the limit of breath holding (45 seconds). Measurements have been made on Subject R.M. on many occasions with the breath held at full inspiration and also at the F.R.C. When the breath is held at the F.R.C. the fall of CO concentration with time is exponential up to the limit of breath holding (about 35 seconds) but with breath holding at full inspiration the fall of CO concentration usually deviates from the straight line at 25 or 30 seconds. This deviation is small in terms of percentage of CO measured and in the experiment illustrated in Figure 7 the concentrations did not vary by more than 0.002 per cent from the values lying on the straight line. This small difference could well be due to an error in calculation of the back pressure of CO in the blood as a result of the recirculation of blood previously exposed to CO in the lungs. These results do not support the findings of Forster and associates (17). Thus, there appears to be no need to postulate variations in the diffusing capacity per unit volume of the lungs of normal subjects, and such a variation of diffusing capacity is unlikely to be the cause of the different results obtained by the single breath and steady state methods of measuring $D_L$. The single breath method has been criticized on the grounds that a sudden inspiration may alter the diffusing capacity of the lungs. The fact that the rate of fall of CO concentration during the first few seconds after inspiration is the same as that throughout the remainder of the breath holding time refutes this criticism.

One of the outstanding differences in the techniques of the single breath and the steady state methods is the lung volume at which the measurements are made. Krogh (18) reported that $D_L$ increased proportionately to alveolar volume at volumes above the "mean capacity." Ogilvie and co-workers (5) found that the increase in $D_L$ was only about 9 per cent for an increase in lung volume of 56 per cent, although three of their five subjects had increases of $D_L$ of 13, 18 and 24 per cent. In the present investigation the mean increase in $D_L$ was 24 per cent when the lung volume increased by 56 per cent. Figure 8 shows the $D_L$ obtained by the single breath and by the steady state methods plotted in relation to the lung volume at which the measurements were made. The lung volume for the steady state measurements is taken as the F.R.C. The steady state values fall approximately on the line relating the single breath values with lung volume; thus, the dependence of $D_L$ on lung volume can explain the whole of the difference between the values obtained for the $D_L$ of normal subjects by the single breath and steady state methods.

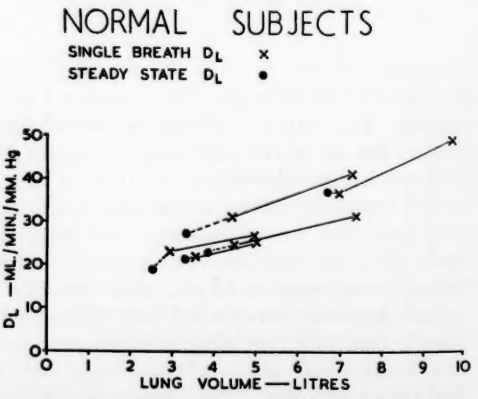



FIG. 8. THE RELATIONSHIP BETWEEN $D_L$, MEASURED BY THE SINGLE BREATH AND STEADY STATE (END TIDAL) METHODS, AND THE LUNG VOLUME AT WHICH THE MEASUREMENTS WERE MADE (NORMAL SUBJECTS)

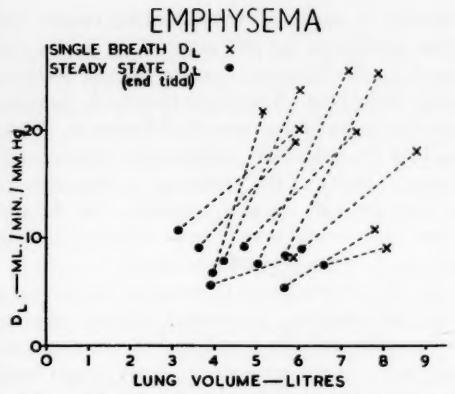



FIG. 9. THE RELATIONSHIP BETWEEN $D_L$, MEASURED BY THE SINGLE BREATH AND STEADY STATE (END TIDAL) METHODS, AND THE LUNG VOLUME AT WHICH THE MEASUREMENTS WERE MADE (PATIENTS WITH EMPHYSEMA)

Other factors, in addition to those discussed above, may account for the different results obtained by the single breath and steady state methods in patients with emphysema. Failure of the alveolar CO concentration to reach a plateau in the expired air in patients with emphysema means that the end tidal sample has no definite value and will vary considerably with the volume expired (Figure 6). The end tidal sample bears no relationship to the mean alveolar concentration of CO which is lower than that of the lowest CO concentration expired during a full expiration. Since the end tidal sample is higher than the mean alveolar CO the value obtained for $D_L$ is lower than the single breath $D_L$. Figure 9 shows the relationship between the single breath and steady state $D_L$, the latter being calculated from the end tidal samples. Some patients with emphysema have a normal $D_L$ by the single breath method but the results are uniformly low by the steady state method. These low values are due, in most cases, to the high end tidal samples and cannot be explained solely by the difference in lung volumes at which the two tests are made.

The steady state $D_L$ calculated from the mean alveolar CO, derived by using a modification of Filley's method, gives values close to those of the single breath $D_L$ (Table VI). The values of $D_L$ obtained by the modification of Filley's steady state method are slightly lower than the single breath $D_L$ in four of the five subjects but the lower value may be due, as in the normal subjects, to the smaller lung volume at which the measurements were made (Figure 10). The agreement between these methods is particularly close when one considers that Filley's method of calculation of the mean alveolar CO is least accurate when used in subjects at rest, in whom the dead space may constitute a large proportion of the tidal volume. This modification of Filley's method allows calculation of the mean end capillary CO instead of the arterial CO which is measured in Filley's original method. The mean alveolar CO calculated is thus uninfluenced by venous shunts and this should bring the measurement of $D_L$ into line with that of $D_{O_2}$ by the method of Riley, Cournand, and Donald (19).

*Physiological implications of the results of fractional measurement of $D_L$*

Previous investigations (8, 20, 21) have shown that in normal subjects the ventilation-perfusion ratio is uniform in fractional samples obtained during a full expiration. Since the ventilation is uniform in normal subjects it is not surprising to find that $D_L$ calculated from the fractional samples is also uniform.

In patients with emphysema, the $D_L$ per unit of lung volume from which the expired air originates

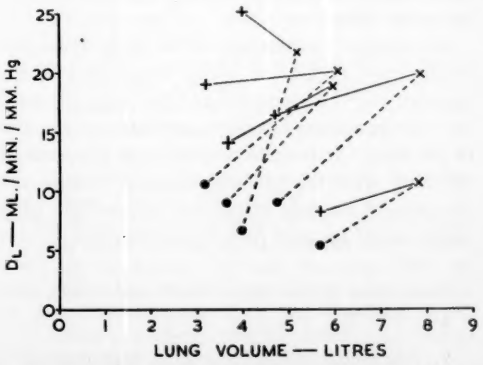



FIG. 10. THE RELATIONSHIP BETWEEN $D_L$, MEASURED BY THE SINGLE BREATH AND THE TWO STEADY STATE METHODS, AND THE LUNG VOLUME AT WHICH THE MEASUREMENTS WERE MADE

×, single breath $D_L$; ●, steady state $D_L$ (end tidal); +, steady state $D_L$ (modification of Filley's method).

is uniform for the different fractions of the expired air. The units of lung volume from which the expired air arises are not necessarily anatomical regions of the lungs, but it is known that the air expired first comes from parts of the lung in which the ventilation-perfusion ratio is high and the air expired last comes from parts of the lung with a low ventilation-perfusion ratio (8, 20). The relationship between $D_L$, the diffusing capacity of the pulmonary membrane $D_M$, the reaction rate of CO with hemoglobin $\theta$, and the capillary blood volume $V_c$ is given by the equation:

$$\frac{1}{D_L} = \frac{1}{D_M} + \frac{1}{\theta V_c}. \qquad (12)$$

A uniform $D_L$ suggests that both $D_M$ and $V_c$ remain uniform in the various regions of the lung. Reduction of $D_M$ in emphysema, when it occurs, most probably is due to a reduction in surface area of the lung and it is unlikely that such a reduction would be accompanied by the increase in $V_c$ which would be necessary to maintain $D_L$ constant. $V_c$ is not necessarily directly related to the perfusion of the lung, but if it were, since $V_c$ per unit lung volume remains uniform throughout the lung one would expect that the variation in the ventilation-perfusion ratio would be the same as the variation in ventilation. This hypothesis is supported by the results shown in Table VII; the ratio of the ventilation in the fractional samples is approximately the same as that of the ventilation-perfusion ratios.

Histological examination of the lungs in emphysema shows regions in which breakdown of the alveolar walls has occurred and this might be used as evidence against a uniform perfusion of all parts of the lung. It is quite possible that the parts of the lung with the greatest alveolar damage are the parts of the lung containing trapped gas (22) which would not take part in gas diffusion.

*Clinical value of the single breath and steady state $D_L$*

*1. The single breath $D_L$.* This measurement is independent of unevenness of ventilation and is affected only by the diffusing capacity of the pulmonary membrane and by the capillary blood volume of the lungs. It is a measure of the potential diffusing capacity of the lungs since the diffusing capacity of parts of the lung which receive very little ventilation has the same effect on the final result as the diffusing capacity of well-ventilated parts of the lung. The single breath $D_L$, therefore, provides a useful measure of diffusion in diseases such as the pulmonary infiltrations where the diffusing capacity of the membrane is impaired; but in diseases with unequal ventilation the diffusing capacity measured may have no relationship to the ability of the lungs to take up oxygen.

*2. The steady state $D_L$ measured by the use of end tidal samples.* In normal subjects or in patients with uniform ventilation of the lungs this test will give the same result as the single breath test when allowance is made for the different lung volumes at which the measurements are made. This test is more time consuming to carry out than the single breath test and has no advantages over the latter test for measurements made at rest. It has the advantage that measurements can be made during exercise and the ability of the subject to increase $D_L$ on exercise may be of clinical importance. When used in patients one must bear in mind that if the ventilation becomes uneven on exercise the measure of $D_L$ by this method will be inaccurate. This test has proved to be of some value clinically and in emphysema it provides a measure which is useful for assessing disability and prognosis (23). The reason for the usefulness of this test is that it measures the combined effect of poor diffusion and unequal ventilation. It is not a measure of diffusion alone when the ventilation is uneven and when applied to patients with uneven ventilation the results should not be expressed as ml. CO per minute per mm. Hg.

*3. The steady state $D_L$ (Filley's method).* This method, modified by the measurement of mixed venous $pCO_2$ instead of arterial $pCO_2$, provides a bloodless method for measuring $D_L$ at rest. With this modification the result is unaffected by venous shunts. The modified method has not been tested during exercise, but it is probably unsuited for use under conditions in which the cardiac output is not even approximately known and in which the circulation time is more rapid. The original method of Filley still provides the only method of measurement of $D_L$ on exercise in patients with unequal distribution of the inspired air.

## SUMMARY

The diffusing capacity of the lungs for CO ($D_L$) has been measured in 5 normal subjects and in 11 patients with emphysema by the single breath method and by the steady state method, using end tidal samples as a measure of the mean alveolar CO tension. In five of the patients with emphysema, $D_L$ was also calculated by a modification of Filley's method.

Fractional samples were taken from a full expiration after breath holding and $D_L$ was calculated for each sample by the single breath method. In normal subjects and in patients with emphysema, $D_L$ was uniform throughout the expired air. Fractional analysis of the expired air of normal subjects in the steady state breathing CO showed that the concentration of CO in the lungs was uniform. In these subjects end tidal samples approximated the mean alveolar concentration of CO and could be used in the calculation of the steady state $D_L$. Measurement of $D_L$ by the single breath and steady state methods gave the same results in normal subjects when allowance was made for the different lung volumes at which the measurements were made.

Patients with emphysema did not have uniform concentrations of CO throughout the lung in the steady state; end tidal samples bore no relationship to the mean alveolar CO and could not be used for calculation of $D_L$.

A bloodless modification of Filley's method of calculation of the steady state $D_L$ is described. This method gave results which were close to those of the single breath $D_L$ measured in the same subjects.

The clinical value of the single breath and steady state methods for measurement of $D_L$ is discussed.



## ACKNOWLEDGMENTS

I am grateful to Miss J. A. West and Miss M. Endacott for skilled technical assistance.


## APPENDIX

*Correction, for time of expiration, of CO samples expired at the end of a period of steady state breathing*

*Example:* Subject, M.E.; correction of the 0.5 liter sample. $D_L$ at F.R.C. equals 21 ml. CO per minute per mm. Hg. Barometric pressure equals 763 mm. Hg. Approximate alveolar volume equals 3.0 liters. Delay of expiration of 0.5 liter sample after end of normal expiration (measured from tracing) equals 0.61 second. Measured concentration of CO in 0.5 liter sample ($F_{E_{CO}}$) equals 0.1628 per cent. From the $D_L$ equation (5):

$$21 = \frac{\text{ln.}\dfrac{F_{IN_{CO}}}{F_{E_{CO}}} \times 3000 \times 60}{0.61 \times 716}.$$

$\text{ln.}\dfrac{F_{IN_{CO}}}{F_{E_{CO}}}$ equals 0.0510. Therefore, $F_{IN_{CO}}$ equals 1.052 times $F_{E_{CO}}$. $F_{IN_{CO}}$ (concentration of CO in sample at time of the end of a normal expiration) equals 1.052 times 0.1628 equals 0.1714 per cent.

## REFERENCES


1. Filley, G. F., MacIntosh, D. J., and Wright, G. W. Carbon monoxide uptake and pulmonary diffusing capacity in normal subjects at rest and during exercise. J. clin. Invest. 1954, 33, 530.
2. Bates, D. V., Boucot, N. G., and Dormer, A. E. The pulmonary diffusing capacity in normal subjects. J. Physiol. 1955, 129, 237.
3. Bates, D. V., and Pearce, J. F. The pulmonary diffusing capacity; a comparison of methods of measurement and a study of the effect of body position. J. Physiol. 1956, 132, 232.
4. Krogh, A., and Krogh, M. On the rate of diffusion of carbonic oxide into the lungs of man. Skand. Arch. Physiol. 1910, 23, 236.
5. Ogilvie, C. M., Forster, R. E., Blakemore, W. S., and Morton, J. W. A standardized breath holding technique for the clinical measurement of the diffusing capacity of the lung for carbon monoxide. J. clin. Invest. 1957, 36, 1.
6. Marks, A., Cugell, D. W., Cadigan, J. B., and Gaensler, E. A. Clinical determination of the diffusion capacity of the lungs. Comparison of methods in normal subjects and patients with "alveolar-capillary block" syndrome. Amer. J. Med. 1957, 22, 51.
7. Linderholm, H. On the significance of CO tension in pulmonary capillary blood for determination of pulmonary diffusing capacity with the steady state CO method. Acta med. scand. 1957, 156, 413.
8. Marshall, R., Bates, D. V., and Christie, R. V. Fractional analysis of the alveolar air in emphysema. Clin. Sci. 1952, 11, 297.
9. Sivertson, S. E., and Fowler, W. S. Expired alveolar carbon dioxide tension in health and in pulmonary emphysema. J. Lab. clin. Med. 1956, 47, 869.
10. Dill, D. B., Edwards, H. T., and Consolazio, W. V. Blood as a physico-chemical system. XI. Man at rest. J. biol. Chem. 1937, 118, 635.
11. Bates, D. V., and Christie, R. V. Intrapulmonary mixing of helium in health and in emphysema. Clin. Sci. 1950, 9, 17.
12. Forster, R. E., Roughton, F. J. W., Cander, L., Briscoe, W. A., and Kreuzer, F. Relationship between diffusing capacity of pulmonary membrane and

pulmonary capillary blood volume in man. Amer. J. Physiol. 1955, **183**, 615.

13. Rahn, H., and Fenn, W. O. A Graphical Analysis of the Respiratory Gas Exchange; The $O_2$–$CO_2$ Diagram. Washington, The American Physiological Society, 1955.
14. Forster, R. E., Fowler, W. S., and Bates, D. V. Considerations on the uptake of carbon monoxide by the lungs. J. clin. Invest. 1954, **33**, 1128.
15. Gréhant, N. Recherches physiques sur la respiration de l'homme. J. Anat. (Paris) 1864, **1**, 532.
16. Dubois, A. B., Britt, A. G., and Fenn, W. O. Alveolar $CO_2$ during the respiratory cycle. J. appl. Physiol. 1952, **4**, 535.
17. Forster, R. E., Fowler, W. S., Bates, D. V., and Van Lingen, B. The absorption of carbon monoxide by the lungs during breathholding. J. clin. Invest. 1954, **33**, 1135.
18. Krogh, M. The diffusion of gases through the lungs of man. J. Physiol. 1915, **49**, 271.
19. Riley, R. L., Cournand, A., and Donald, K. W. Analysis of factors affecting partial pressures of oxygen and carbon dioxide in gas and blood of lungs: Methods. J. appl. Physiol. 1951, **4**, 102.
20. Roelsen, E. The composition of the alveolar air investigated by fractional sampling: Comparative investigations on normal persons and patients with bronchial asthma and pulmonary emphysema. Acta med. scand. 1939, **98**, 141.
21. Cotton, F. S. Studies in respiration; I. A simple method for the investigation of fractional samples of expired air. Austr. J. exp. Biol. med. Sci. 1939, **17**, 425.
22. Bedell, G. N., Marshall, R., DuBois, A. B., and Comroe, J. H., Jr. Plethysmographic determination of the volume of gas trapped in the lungs. J. clin. Invest. 1956, **35**, 664.
23. Bates, D. V., Knott, J. M. S., and Christie, R. V. Respiratory function in emphysema in relation to prognosis. Quart. J. Med. 1956, **25**, 137.

# EVALUATION OF CATECHOL AMINE LEVELS IN RENAL INSUFFICIENCY

By M. SEREF ZILELI, JAMES T. HAMLIN, III, FRITZ W. REUTTER, AND DALE G. FRIEND

*(From the Departments of Medicine, Harvard Medical School and Peter Bent Brigham Hospital, Boston, Mass.)*

(Submitted for publication August 28, 1957; accepted November 14, 1957)

The estimation of epinephrine (E) and norepinephrine (NE) in plasma and urine has become important as a procedure in physiological research and as an aid in the diagnosis of pheochromocytoma. With the development of fluorometric procedures the chemical estimation of E and NE in biological fluids and tissues was made possible.

The fluorometric procedures used most commonly are modifications of the trihydroxyindole method of Lund (1) and the ethylenediamine (EDA) condensation method of Weil-Malherbe and Bone (2, 3). The trihydroxyindole method is not sensitive enough to permit measurements of E and NE in small amounts of normal peripheral plasma (1), and its practical usefulness is limited to the assay of urine. The sensitivity of the EDA condensation method is such that extremely small amounts of E and NE can be measured; however, it is not as specific as the trihydroxyindole method and will detect phenolic compounds other than E and NE (4).

While employing the EDA condensation method as an aid in the diagnosis of pheochromocytoma, we have observed, in some individuals with renal insufficiency who later were found not to have a pheochromocytoma, levels of E and NE much higher than those which occur in normal patients. The purpose of the present study was to determine whether the high levels of E and NE frequently observed in patients with renal insufficiency are true values or are due to the presence of substances which interfere with the EDA method and produce false high values of E and NE.

The presence of large amounts of these substances may be due to an abnormal or increased production of E, NE or interfering compounds, or an inability on the part of the kidneys to excrete the normal amounts of these substances present in the blood. Among possible interfering substances which occur in abnormal amounts in the blood of patients with renal insufficiency are urea, creatinine, uric acid and phenolic compounds. Of these, the phenolic compounds are most likely to be involved.

The phenols are excreted in both free and conjugated forms, with a normal daily output in the urine of 200 to 500 mg. (5). They are derived mainly from bacterial putrefaction of aromatic amino acids in the intestine. A minor portion comes from the presence of sex hormones, epinephrine, norepinephrine and tyrosine in the blood. The normal blood levels calculated as phenol range from 1 to 2 mg. per 100 ml., whereas in uremic patients they may reach 6 to 10 mg. per 100 ml. (6). The importance of these compounds in the pathogenesis of uremic symptoms has been reviewed by Harrison and Mason (7).

In order to determine whether the presence of large amounts of phenolic compounds in the plasma of patients with renal insufficiency can account for the high levels of E and NE as measured by the EDA condensation method, the effect of various phenolic compounds on this method and the trihydroxyindole method was investigated, and the specificity of these two fluorometric procedures for estimating catechol amines was compared. Plasma E and NE levels were assayed by both methods in normal individuals and patients with renal disease. The high E and NE levels found in the patients with renal insufficiency were then evaluated in the light of information developed from the differences in specificity established for the two methods.

## METHODS

Standard solutions containing monohydroxy-, dihydroxy- and trihydroxyphenyl compounds which might be expected to occur in the blood were prepared. Known amounts of these were added to pooled heparinized plasma for recovery determinations. Extraction from plasma

TABLE I

*Compounds which produce fluorescence with ethylenediamine and are adsorbed from plasma by alumina*

| Compound | Amount added (μg.) | Relative fluorescent intensity* 485 mμ | 600 mμ | Recovery (%) |
|---|---|---|---|---|
| Norepinephrine | 0.2 | 100 | 40 | 82 |
| Epinephrine | 0.2 | 18 | 100 | 98 |
| Isoprenaline | 0.2 | 45 | 100 | 90 |
| Dopac | 0.2 | 23 | 8 | 95 |
| Dopamine | 0.2 | 6.1 | 35 | 88 |
| Dopa | 50.0 | 85 | 209 | 65 |
| Catechol | 0.2 | 227 | 100 | 86 |
| Phenol | 0.2 | 14 | 7 | 30 |
| Pyrogallol | 0.2 | 3.6 | 25 | 50 |

* Relative to fluorescence of norepinephrine at 485 mμ and epinephrine at 600 mμ.

was accomplished by adsorption on activated alumina at pH 8.0 to 8.5, followed by elution with acetic acid. An Aminco-Bowman spectrophotofluorometer was used to measure fluorescence.

Blood specimens were drawn into heparinized tubes, centrifuged, and the plasma removed and immediately frozen. Five ml. specimens of plasma were used for assays by the EDA condensation method. The lower degree of sensitivity of the trihydroxyindole method required larger plasma specimens for accurate determinations. One hundred ml. of plasma was used for each determination in normal individuals and 30 ml. of plasma for assays in patients with renal disease.

*Ethylenediamine method.* The method of Weil-Malherbe and Bone (2, 3), as modified by Aronow and Howard (8), was used. Fluorescence of the condensation fluorophors was determined using an excitation wave length of 430 mμ and fluorescent wave lengths of 485 and 600 mμ for NE and E, respectively. When the condensation fluorophor of E is activated at 430 mμ it produces a fluorescent peak at 520 mμ. However, in determining E in solutions containing both E and NE, fluorescence at 600 mμ is used because at this wave length the fluorescence of E exceeds that of NE, and the separation of their respective fluorescent curves is maximal.

*Trihydroxyindole method.* The method of Lund (1), as modified by von Euler and Floding (9), was used. The intensities of fluorescence were determined using an excitation wave length of 415 mμ and a fluorescent wave length of 515 mμ.

## RESULTS

### *Ethylenediamine method*

Standard solutions of the phenolic compounds, epinephrine, norepinephrine, isoprenaline (3,4-dihydroxyphenyl-β-isopropyl-aminoethanol), dopa (3,4-dihydroxyphenyl-β-aminopropanoic acid), dopamine (3,4-dihydroxyphenyl-β-aminoethane), dopac (3,4-dihydroxyphenyl acetic acid), phenol, catechol, pyrogallol, *o*-cresol, *p*-cresol, phloroglucinol, quinol, tyrosine and serotonin (5-hydroxytryptamine) form fluorescent condensation products with ethylenediamine. Of these, only E, NE, isoprenaline, dopa, dopamine, dopac, phenol, catechol and pyrogallol are adsorbed from plasma by alumina. Recoveries of these compounds after addition of known amounts to plasma and assay by the EDA method are shown in Table I. Phenolic compounds which produce fluorescence with EDA but are not adsorbed from plasma by alumina are shown in Table II.

The fluorescent spectra of each of the compounds recovered from plasma were determined using an excitation wave length of 430 mμ. Dopac, catechol and phenol were found to have fluorescent maxima at 485, 485, and 480 mμ, respectively. These compounds, when present, will interfere mainly with the determination of NE. Dopa, dopamine, isoprenaline and pyrogallol have fluorescent maxima at 520 mμ, the wave length of maximal fluorescence of E. Therefore, if present in plasma, they will interfere mainly with the estimation of E. However, because of some overlap of the fluorescent spectral curves of these compounds with those of both E and NE, the presence of any of the compounds will interfere in the determination of either E or NE.

Creatinine, urea, uric acid, indole and phosphate, all of which are found in increased quantities in the blood of uremic patients, were tested with the EDA reaction. Creatinine was the only one of these compounds that produced fluorescence; however, it is not adsorbed by alumina and therefore will not interfere with the determination of E and NE.

TABLE II

*Compounds which produce fluorescence with ethylenediamine but are not adsorbed from plasma by alumina*

| Compound | Amount added (μg.) | Relative fluorescent intensity* 485 mμ | 600 mμ | Recovery (%) |
|---|---|---|---|---|
| *p*-cresol | 400 | 13 | 24 | 0 |
| *o*-cresol | 100 | 4 | 3 | 0 |
| Phloroglucinol | 100 | 32 | 95 | 0 |
| Quinol | 0.2 | 100 | 90 | 0 |
| Serotonin | 20 | 100 | 144 | 0 |

* Relative to fluorescence of norepinephrine at 485 mμ and epinephrine at 600 mμ.

TABLE III

*Plasma epinephrine and norepinephrine levels in patients with renal insufficiency as determined by the ethylenediamine condensation method*

| Patient | NE | E | Blood urea nitrogen |
|---|---|---|---|
| | *μg. per liter plasma* | | *mg. per 100 ml.* |
| E. R. | 8.3 | 0.8 | 220 |
| B. J. | 8.1 | 1.0 | 187 |
| R. B. | 6.5 | 1.5 | 159 |
| I. L. | 7.7 | 0.6 | 96 |
| M. V. | 3.4 | 3.8 | 55 |
| R. R. | 15.0 | 7.2 | 135 |
| M. O. | 8.4 | 1.4 | 150 |
| J. P. | 12.5 | 5.5 | 120 |
| B. F. | 15.1 | 1.9 | 95 |
| U. P. | 9.7 | 0.4 | 90 |

Plasma E and NE, as determined by the EDA method in a series of patients with renal insufficiency, are shown in Table III. In three of these patients both the E and NE values were elevated above the normal range. Six patients showed abnormal levels of the NE fraction alone, while one patient showed an abnormal elevation only in the E fraction. There was no correlation between the blood-urea-nitrogen levels and the level of elevation of E or NE.

In three patients determinations were made before and after dialysis on the artificial kidney (Table IV). In two of these cases the elevated values before dialysis became normal following dialysis. In the third patient (E.H.) the NE level dropped to normal after dialysis but the E level remained elevated.

TABLE IV

*Plasma epinephrine and norepinephrine levels in uremic patients before and after dialysis on the artificial kidney as determined by the ethylenediamine condensation method*

| Patient | NE | E | Blood urea nitrogen |
|---|---|---|---|
| | *μg. per liter plasma* | | *mg. per 100 ml.* |
| E. H. | | | |
| Before dialysis | 13.0 | 3.8 | 200 |
| After dialysis | 5.2 | 2.4 | 120 |
| S. R. | | | |
| Before dialysis | 4.1 | 3.2 | 150 |
| After dialysis | 3.5 | 0.3 | 75 |
| F. S. | | | |
| Before dialysis | 8.6 | 0.0 | |
| After dialysis | 5.8 | 0.0 | |

TABLE V

*Trihydroxyindole method: Compounds which produce fluorescence*

| Compound | Amount added (μg.) | % oxidation | | Relative fluorescent intensity |
|---|---|---|---|---|
| | | pH 3.5 | pH 6.5 | |
| Epinephrine | 1.0 | 100 | 100 | 100 |
| Norepinephrine | 1.0 | 4 | 100 | 73.6 |
| Isoprenaline | 1.0 | 100 | 100 | 136 |
| Dopamine | 10.0 | | | 0.5* |

* Fluorescence occurs only at pH 6.5.

### *Trihydroxyindole method*

Epinephrine, norepinephrine, isoprenaline and, to a limited extent, dopamine, produced fluorescence with the trihydroxyindole method (Table V). Isoprenaline is completely oxidized at both pH 3.5 and 6.5 and will thus interfere with the determination of E only. Isoprenaline has a relative fluorescent intensity equal to E when activated at 415 mμ with a fluorescence determined at 515 mμ. If isoprenaline is activated at 350 mμ, it will produce a fluorescent peak at 520 mμ, whereas E and NE have no fluorescence when excited at 350 mμ. Therefore, any interference due to the presence of isoprenaline could be detected in this manner.

Plasma E and NE levels as determined by both the EDA condensation and the trihydroxyindole methods in six normal individuals and five uremic patients are shown in Table VI. NE levels deter-

TABLE VI

*Comparison of the ethylenediamine (EDA) condensation and the trihydroxyindole (THI) methods in normal individuals and patients with renal insufficiency*

| Subject | EDA method | | THI method | |
|---|---|---|---|---|
| | NE | E | NE | E |
| | *μg. per liter plasma* | | *μg. per liter plasma* | |
| Normal | | | | |
| F. M. | 3.8 | 0.8 | 3.2 | 0.7 |
| E. R. | 2.2 | 0.6 | 2.0 | 0.1 |
| J. B. | 2.1 | 0.9 | 1.8 | 0.1 |
| F. R. | 2.6 | 0.4 | 1.7 | 0.1 |
| J. R. | 3.3 | 1.3 | 2.4 | 0.1 |
| J. F. | 2.2 | 0.3 | 2.1 | 0.1 |
| Renal disease | | | | |
| V. R. | 6.5 | 0.6 | 2.1 | 0.0 |
| U. F. | 6.2 | 0.9 | 1.5 | 0.9 |
| J. T. | 8.7 | 0.0 | 3.0 | 0.0 |
| M. S. | 15.1 | 0.0 | 2.6 | 0.15 |
| L. O. | 8.4 | 1.4 | 2.2 | 0.0 |

mined by the EDA method were elevated in each uremic case; however, the E levels in these five patients were within the normal range. Both NE and E were within the normal range when determined by the trihydroxyindole method.

*Sensitivity and normal values*

Addition and recovery experiments with the EDA method indicated that 0.08 μg. per liter of E and NE can be determined from eluates of 5 ml. of plasma. With this size plasma sample, 1 μg. per liter was the lowest concentration accurately determined by the trihydroxyindole method; therefore, larger specimens of plasma were used for this method as described above. In thirty normal subjects plasma E and NE determined by the EDA method gave a mean value for E of 0.47 μg. per liter plasma, with a range from 0 to 1.5; for NE the mean value was 2.72 μg. per liter plasma, with a range from 1.5 to 4.5. In six normal subjects plasma E and NE determined by the trihydroxyindole method gave a mean value for E of 0.2 μg. per liter plasma, with a range from 0.0 to 0.7; for NE the mean value was 2.2 μg. per liter plasma, with a range from 1.7 to 3.2.

## DISCUSSION

The results indicate that the EDA reaction is not specific for E and NE. All of the phenol compounds tested produce fluorophors with EDA whose spectra overlap the spectra of E and NE. Therefore, if they are present in the blood, they could interfere with the fluorimetric determination of E and NE. However, only E, NE, isoprenaline, dopa, dopamine, dopac, phenol, catechol and pyrogallol are adsorbed from plasma by alumina.

The adsorptive properties of aluminum oxide, although not specific for the dihydroxy compounds as has been stated by von Euler and Orwen (10), limit the number of possible interfering substances for the EDA method. Phenol is the only monohydroxy compound that was adsorbed. Of the dihydroxy and trihydroxy compounds tested only those having a hydroxyl group in the ortho position were adsorbed by alumina.

Isoprenaline has been shown by paper chromatography to be present in the human adrenal medulla (11), but it has not been found in blood or urine. Dopamine (12) and dopac (13) have been identified in the urine and thus presumably are present in blood. Dopa has not been proved to be present in biological fluids. Other phenols occur in both blood and urine.

In the patient with normal renal function, the amount of interference from these substances, if present, would be small and relatively constant. However, in patients with renal insufficiency their excretion may be decreased with a resultant elevation in blood level. The presence of these or other interfering compounds in the blood could account for the high catechol amine levels observed in these patients.

The fact that the interference does not follow a definite pattern, occurring in both E and NE fractions in some cases and in the E or the NE fraction alone in other cases, suggests that more than one interfering substance is present. No single substance could account for these variations in interference. It is evident from the results obtained in patients with renal disease that the interfering substances are dialysable and that there is no relation between the degree of interference and the blood-urea-nitrogen levels. Although the identity of the interfering substances in these cases has not been established, it is possible that some of the compounds listed in Table I are involved.

The formation of fluorophors in the trihydroxyindole method requires the closure of the indole ring. Only isoprenaline was found to interfere to any significant degree with the estimation of E and NE by this method. Since this substance has only been identified in the adrenal medulla where it constitutes less than one per cent of the total catechol amine present, the trihydroxyindole method can be considered a highly specific method of estimating E and NE.

In the five patients with renal insufficiency, all of whom had elevated NE levels with the EDA method, the plasma E and NE were both within the normal range when determined by the trihydroxyindole method. Therefore, the substances present in the blood of uremic patients which interfere with the EDA method do not interfere with the trihydroxyindole method. In six normal individuals the plasma E and NE values determined by the trihydroxyindole method were slightly lower than those determined by the EDA method. The difference in values found in normal persons by the two methods, however, was not sufficient

to account for the apparent differences in values found by these methods on plasma from patients with renal disease. This is further evidence that the elevated levels of E and NE determined by the EDA method in plasma of patients with renal insufficiency do not represent true values for E and NE but are due to the presence of interfering substances.

Valk and Price have recently shown that many of the phenolic compounds present in the blood and urine are capable of interfering with the EDA method (4). They were able to show a significant difference between normal plasma E and NE values determined by the EDA as opposed to the trihydroxyindole method. Although our results show that various phenolic compounds can interfere with the EDA method, we did not find a significant difference between the E and NE levels determined by the two methods in plasma of normal individuals. The discrepancies between the normal values by the THI method reported by Valk and Price and ourselves may be explained by the fact that these authors used smaller plasma samples for their determinations. The sensitivity of the THI method is such that there may be considerable error introduced if small plasma samples are employed. Our findings in plasma of normal subjects are in agreement with those of Weil-Malherbe, who reports identical results with both methods (14).

## SUMMARY AND CONCLUSIONS

1. The high plasma epinephrine and norepinephrine levels determined by the ethylenediamine condensation method in patients with renal insufficiency are not true values for epinephrine and norepinephrine and are due to the presence of substances in the blood of these patients which interfere with this method of assay.

2. The identity of the interfering substances has not been established; however, the phenolic compounds which occur in large quantities in the blood of patients with renal insufficiency may be involved.

3. The substances which interfere with the ethylenediamine determinations do not affect the determinations made with the highly specific trihydroxyindole method.

## REFERENCES

1. Lund, A. Simultaneous fluorimetric determinations of adrenaline and noradrenaline in blood. Acta pharmacol. (Kbh.) 1950, **6**, 137.
2. Weil-Malherbe, H., and Bone, A. D. The chemical estimation of adrenaline-like substances in blood. Biochem. J. 1952, **51**, 311.
3. Weil-Malherbe, H., and Bone, A. D. The adrenergic amines of human blood. Lancet 1953, **1**, 974.
4. Valk, A. de T., Jr., and Price, H. L. The chemical estimation of epinephrine and norepinephrine in human and canine plasma. I. A critique of the ethylenediamine condensation method. J. clin. Invest. 1956, **35**, 837.
5. Williams, R. T. Detoxication Mechanisms. The Metabolism of Drugs and Allied Organic Compounds. New York, John Wiley & Sons Inc., 1947, p. 73.
6. Hoffman, W. S. The Biochemistry of Clinical Medicine. Chicago, The Yearbook Publishers, Inc., 1954, p. 279.
7. Harrison, T. R., and Mason, M. F. The pathogenesis of the uremic syndrome. Medicine 1937, **16**, 1.
8. Aronow, L., and Howard, F. A. Improved fluorometric technique to measure changes in adrenal epinephrine-norepinephrine output caused by veratrum alkaloids. Fed. Proc. 1955, **14**, 315.
9. von Euler, U. S., and Floding, I. A fluorimetric micromethod for differential estimation of adrenaline and noradrenaline. Acta physiol. scand. 1955, **33**, suppl. **118**, 45.
10. von Euler, U. S., and Orwen, I. Preparation of extracts of urine and organs for estimation of free and conjugated noradrenaline and adrenaline. Acta physiol. scand. 1955, **33**, suppl. **118**, 1.
11. Lockett, M. F. Identification of an isoprenaline-like substance in extracts of adrenal glands. Brit. J. Pharmacol. 1954, **9**, 498.
12. von Euler, U. S., and Hellner, S. Excretion of noradrenaline, adrenaline and hydroxytyramine in urine. Acta physiol. scand. 1951, **22**, 161.
13. von Euler, C., von Euler, U. S., and Floding, I. Biologically inactive catechol derivatives in urine. Acta physiol. scand. 1955, **33**, suppl. **118**, 32.
14. Weil-Malherbe, H. The fluorimetric estimation of adrenaline and noradrenaline in plasma. J. Physiol. 1957, **135**, 44P.

# AMMONIUM UPTAKE BY THE EXTREMITIES AND BRAIN IN HEPATIC COMA [1, 2]

By LESLIE T. WEBSTER, JR.,[3] AND GEORGE J. GABUZDA

*(From the Department of Medicine, Western Reserve University School of Medicine at City Hospital, Cleveland, Ohio)*

(Submitted for publication February 11, 1957; accepted November 14, 1957)

Certain compounds containing or liberating ammonium may induce the syndrome of impending hepatic coma in susceptible patients with hepatic cirrhosis or Eck fistula (1–4). This syndrome is often but not always associated with increased ammonium concentrations in the blood (5, 6).

Although the origin and sources of elevated blood ammonium concentrations are not entirely clear, several factors contributing to them have been investigated. Decreased ammonium removal by the liver, resulting from either inadequate urea synthesis or from portal-systemic shunting of blood (7–10), is an important factor since increased arterial blood ammonium concentrations rarely occur if the liver and its circulation are normal. Elevated blood ammonium concentrations from an increased ammonium load are also observed in patients with liver disease. An increased load may result from the oral or intravenous administration of ammonium salts, amino acids and urea (1, 2, 11–13), from protein given orally (3, 14), and from gastrointestinal bleeding (15).

Previous investigations of patients with hepatic decompensation have demonstrated that the extremities and brain, which contain systems active in ammonium metabolism, are important sites for the removal of ammonium from the circulating blood (16–18). The roles of these peripheral sites in ammonium metabolism were evaluated in the present study also. In some instances, the extremities and brain of patients with severe hepatic disease were found to remove significant amounts of ammonium from a given volume of blood. However, in other instances they failed to do so, and thereby contributed indirectly to the increased blood ammonium concentrations observed.

## METHODS

Three groups of patients were studied. The first consisted of 24 hospitalized patients without liver disease, the second of 27 patients with hepatic cirrhosis, and the third of 23 patients with hepatic cirrhosis and either impending hepatic [4] coma or coma. The last group is referred to subsequently as the "coma" group. Patients in the "coma" group were divided into one group without an added nitrogen load and a second group of patients with an added nitrogen load in the form of blood in their gastrointestinal tracts (evidence of upper gastrointestinal hemorrhage or massive epistaxis and blood swallowing). Pertinent clinical and laboratory data on all patients are presented in Tables I, II and III.

Patients were observed on the wards of Cleveland City Hospital, Crile Veterans Administration Hospital, and Boston City Hospital.[5] Confusion, coma and "flapping" tremor were graded from first to fourth degree; first degree indicated a mild, and fourth degree a serious, disturbance for each condition as previously described (20). Estimated daily protein intakes ranged from 30 to 100 grams for patients not in the "coma" group. Protein was withheld from confused or comatose patients for at least 24 and usually more than 48 hours before blood sampling.

Control was instituted over most factors known to increase blood ammonium ($NH_4$-N) concentrations in patients with hepatic disease. No patient received ammonium salts within 72 hours of blood sampling. Blood samples were obtained only when patients had not received nitrogenous foods for the preceding 12 hours (13). Active gastrointestinal bleeding and epistaxis (15) were absent in all patients except some in the "coma" group. Because urea given intravenously may cause prompt increases in blood $NH_4$-N concentrations (13), patients

[1] This investigation was supported in part by the Research and Development Division, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-749.

[2] A report of this investigation was presented at the Twenty-Ninth Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., November 9, 1956 (J. Lab. clin. Med. 1956, **48**, 954).

[3] Russell M. Wilder Fellow of the National Vitamin Foundation.

[4] Characterized by mental confusion and a "flapping" tremor (19).

[5] Five patients in the " coma" group were studied at Boston City Hospital. Six patients in the cirrhosis group and one in the " coma" group were observed at Crile Veterans Administration Hospital in Cleveland.

TABLE I

*Clinical and laboratory data in 24 patients without liver disease*

| Patient | Age and sex | Diagnosis | Serum total bilirubin | Serum BSP | B.U.N. | Blood $NH_4$-N (*micrograms per 100 ml.*) Arm vein | Artery | Jugular bulb | A-V diff. extremities | A-V diff. brain |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mg./100 ml.* | *%* | *mg./100 ml.* | | | | | |
| 1 | 68 M | Remote C.V.A.* | 0.2 | 0 | 10 | 48 | 50 | 43 | + 2 | + 7 |
| 2 | 67 M | Remote C.V.A.* | 0.5 | | 22 | 47 | 26 | 52 | −21 | −26 |
| 3 | 42 M | Myotonia dystrophica | 0.2 | 2 | 12 | 27 | 46 | 43 | +19 | + 3 |
| 4† | 65 M | Lues, ?pneumonia | 0.6 | 7 | 17 | 55 | 55 | 60 | 0 | − 5 |
| 5 | 75 M | Senility | 0.1 | 0 | 21 | 73 | 86 | 78 | +13 | + 8 |
| 6 | 65 M | Peptic ulcer | 1.8 | 2 | 17 | 65 | 54 | | −11 | |
| 7 | 44 M | Peptic ulcer | 0.3 | 1 | 12 | 46 | 49 | | + 3 | |
| 8 | 70 F | Hypertension | 0.7 | 7 | 22 | | 61 | 49 | | +12 |
| 9 | 82 M | Senility | 0.2 | 13 | 16 | | 40 | 52 | | −12 |
| 10 | 74 M | Emphysema | 0.3 | 9 | 8 | 46 | 47 | 60 | + 1 | −13 |
| 11† | 62 M | ?Bronchogenic carcinoma | 0.7 | 2 | 18 | 35 | 28 | 43 | − 7 | −15 |
| 12 | 36 F | ?Cushing's disease | 0.5 | | 10 | 35 | 34 | 50 | − 1 | −16 |
| 13 | 63 M | Hypertension, fibrosarcoma | 0.9 | 4 | 21 | 44 | 40 | 44 | − 4 | − 4 |
| 14† | 26 M | Pneumonia | 0.6 | 0 | 11 | 40 | 41 | 43 | + 1 | − 2 |
| 15 | 42 M | Peptic ulcer | 0.1 | 9 | 13 | 37 | 45 | 48 | + 8 | − 3 |
| 16 | 52 M | Hypertension | 0.5 | 8 | 14 | 43 | 38 | 41 | − 5 | − 3 |
| 17 | 41 M | Hypertension | | 4 | 23 | 32 | 49 | 43 | +17 | + 6 |
| 18‡ | 42 F | Subsided pyelonephritis | | 3 | 20 | 22 | 21 | 23 | − 1 | − 2 |
| 19 | 28 F | Undiagnosed fever | 0.6 | 8 | 21 | 23 | 22 | 27 | − 1 | − 5 |
| 20 | 67 M | ?Pulmonary infarct | 0.7 | 7 | 24 | 49 | 43 | 35 | − 6 | + 8 |
| 21§ | 32 M | Lung abscess | 0.1 | 1 | 10 | 25 | 47 | 44 | +22 | + 3 |
| 22‖ | 42 F | Subsided pyelonephritis | | | 10 | 22 | 34 | 30 | +12 | + 4 |
| 23 | 35 M | Peptic ulcer | | 3 | 14 | 57 | 51 | 54 | − 6 | − 3 |
| 24 | 45 M | Emphysema | 0.3 | 2 | 18 | 60 | 57 | 62 | − 3 | − 5 |

* Cerebral vascular accident.
† Penicillin discontinued for at least 72 hours in Patients 4, 11 and 14.
‡ Gantrisin® discontinued for three days in Patient 18.
§ Patient 21 receiving penicillin.
‖ Patient 22 receiving Panmycin® and nitrofuridantin.

in the normal and cirrhosis groups were selected so that none had elevated blood urea nitrogen concentrations. Patients having clinical evidence of shock or a systolic blood pressure below 90 mm. Hg were not included because animal experiments indicate that shock may produce increased blood $NH_4$-N concentrations (21). When steroids, acetazolamide (Diamox®), analgesics or sedatives were given it is so indicated in the tables because some of these drugs have been used in the treatment of impending hepatic coma or have been implicated in its production. The patients studied received neither glutamic acid nor arginine which may lower blood $NH_4$-N concentrations (20, 22–25). Neomycin and tetracycline antibiotics also may lower blood $NH_4$-N concentrations in some patients with hepatic disease (26). Accordingly, antibiotics were used only as mentioned in Tables I, II and III.

Ammonium metabolism in extremities and brain was evaluated by analyzing $NH_4$-N in blood samples obtained within three minutes of each other from a vein located in an extremity, an artery, and a superior jugular bulb. The uptake or release of $NH_4$-N per given volume of blood circulating through the extremity and brain was obtained by calculating the arteriovenous and arteriojugular bulb blood $NH_4$-N concentration differences.

Extremity venous blood was obtained from a forearm vein except as indicated for some patients in the coma group (Table III). The jugular vein was punctured, either in its superior bulb or just below this site, by directing a two inch number 20 gauge lumbar puncture needle with the bevel upwards superiorly and posteriorly from a site just under the ear in the space anterior to the mastoid process and posterior to the mandibular ramus.

Ammonium-nitrogen[6] in blood samples was determined by a modified Conway microdiffusion technique (27) with diffusion times of either 10 or 16⅔ minutes. In all but a few instances the values for $NH_4$-N concentration represent the average of triplicate determinations for a given sample. Concentrations were calculated as recommended by Conway and were expressed as micrograms of $NH_4$-N per 100 ml. of blood. The percentage of nitrogen recovered from a standard ammonium sulfate solution was used to estimate the per cent recovery of volatile base from blood. A modification was introduced

[6] Whether ammonium normally is present in circulating blood or whether it is formed only after blood is shed has not been established. Volatile base, released from blood upon addition of alkali, is assumed to be nearly all ammonium.

at Cleveland, *i.e.*, the rim of the Conway dish was greased rather than the entire underside of the cover glass. This permitted a greater recovery from an ammonium sulfate standard.

The ranges of blood $NH_4$–N concentrations obtained in patients with and without liver disease were comparable in Cleveland and Boston. The reproducibility of $NH_4$–N concentrations in two different blood samples drawn during the day from the same site in a given patient ranged from 0 to 23 micrograms per 100 ml. (mean, 6; S.D., plus or minus 5.2). This range of reproducibility was not significantly different in patients without liver disease compared to those with cirrhosis ($p > 0.7$). Reproducibility in confused or comatose patients with cirrhosis was not tested.

Bromsulphalein concentration was determined in the serum 45 minutes after injection of 5 mg. of this dye per kilogram of body weight (28). Serum bilirubin concentrations were performed by the standard technique (29). The methods of either Myers (30) or of Owings and Mandel (31) were employed for the determination of blood urea nitrogen.

The statistical evaluations presented were derived by the methods described in Snedecor's textbook (32).[7]

## RESULTS

### *Blood ammonium-nitrogen concentrations*

The $NH_4$–N concentrations in blood obtained from different sites in the patient groups studied

[7] Dr. George F. Badger kindly reviewed the application of these statistical methods.

TABLE II

*Clinical and laboratory data in 27 patients with hepatic cirrhosis*

| Patient | Age and sex | Biopsy or autopsy | Physical examination* | Serum total bilirubin | Serum BSP | B.U.N. | Blood $NH_4$-N (*micrograms per 100 ml.*) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Arm vein | Artery | Jugular bulb | A-V diff. extremities | A-V diff. brain |
| | | | | *mg./100 ml.* | % | *mg./100 ml.* | | | | | |
| 1 | 33 F | No | Angiomata, ascites, edema | 1.0 | >20 | 8 | 47 | 70 | 76 | +23 | − 6 |
| 2 | 68 M | No | Ascites, edema | 1.5 | 32 | 15 | 23 | 35 | 53 | +12 | −18 |
| 3 | 55 M | Yes | | 1.1 | 35 | 11 | 21 | 30 | 30 | + 9 | 0 |
| 4 | 49 M | Yes | Angiomata, edema | 1.4 | >20 | 8 | 19 | 29 | 47 | +10 | −18 |
| 5 | 68 M | Yes | Angiomata, ascites, edema | 1.3 | >20 | 17 | 53 | 89 | 75 | +36 | +14 |
| 6 | 40 M | Yes | Angiomata, ascites, edema | 19.7 | | 12 | 39 | 65 | 76 | +26 | −11 |
| 7 | 54 F | No | Ascites, edema | 2.8 | 30 | 5 | 35 | 59 | 58 | +24 | + 1 |
| 8† | 39 F | Yes | | 6.1 | 27 | 13 | 41 | 66 | 65 | +25 | + 1 |
| 9† | 41 M | Yes | Ascites | 0.4 | 7 | 16 | 23 | 39 | 40 | +16 | − 1 |
| 10 | 48 M | No | Angiomata | 6.2 | 52 | 9 | 43 | 92 | 95 | +49 | − 3 |
| 11 | 62 M | Yes | Ascites, edema | 1.2 | 21 | 17 | 31 | 54 | 53 | +23 | + 1 |
| 12 | 68 M | Yes | Ascites, edema | 1.3 | 58 | 14 | 64 | 104 | 88 | +40 | +16 |
| 13 | 57 F | No | Ascites, edema | 4.9 | | 6 | 35 | 87 | | +52 | |
| 14 | 45 F | No | | 0.9 | 16 | 6 | 35 | 60 | | +25 | |
| 15 | 44 F | No | Splenomegaly | 1.2 | 13 | 9 | 78 | 87 | 86 | + 9 | + 1 |
| 16 | 58 M | Yes | Angiomata, ascites, splenomegaly | 2.0 | 38 | 13 | 59 | 67 | 68 | + 8 | − 1 |
| 17 | 63 M | Yes | Angiomata | 5.6 | 23 | 8 | 36 | 37 | 60 | + 1 | −23 |
| 18‡ | 72 M | Yes | | 0.8 | 9 | 19 | 45 | 49 | 48 | + 4 | + 1 |
| 19 | 58 F | Yes | Angiomata, ascites | 0.3 | 23 | 11 | 78 | 91 | 78 | +13 | +13 |
| 20 | 49 M | Yes | Angiomata, ascites, edema | 11.0 | 53 | 12 | 80 | 86 | | + 6 | |
| 21 | 42 F | No | Angiomata, ascites | 1.8 | 22 | 10 | 82 | 93 | 85 | +11 | + 8 |
| 22 | 76 M | Yes | Angiomata, splenomegaly | 0.9 | 34 | 20 | 77 | 79 | 78 | + 2 | + 1 |
| 23 | 71 M | No | Edema | 1.4 | 23 | 15 | 80 | 94 | 98 | +14 | − 4 |
| 24 | 58 M | Yes | Angiomata, splenomegaly | 0.9 | 27 | 13 | 83 | 106 | 120 | +23 | −14 |
| 25 | 56 M | No | Angiomata, ascites, edema | 2.5 | | 20 | 84 | 89 | | + 4 | |
| 26§ | 67 M | Yes‖ | Ascites, edema | 0.9 | 27 | 15 | 69 | 128 | 96 | | +32 |
| 27 | 51 F | Yes | Splenomegaly, edema | 1.8 | 51 | 8 | | 86 | 84 | | + 2 |

* All patients had hepatomegaly except Patients 7 and 11.

† Penicillin was discontinued 72 hours prior to blood sampling in Patients 8 and 9.

‡ Patient 18, with miliary tuberculosis, had been receiving streptomycin, isoniazid and *p*-aminosalicylic acid for months.

§ Patient 26, with pulmonary tuberculosis, was receiving streptomycin. The $NH_4$-N uptake in the extremities was not calculated for Patient 26 because the venous blood sample was not drawn simultaneously with those from the artery and jugular bulb.

‖ Metastatic carcinoma with inferior vena caval obstruction in addition to cirrhosis.

TABLE II
Clinical and laboratory data in 23 patients with cirrhosis and hepatic coma*

TABLE III

*Clinical and laboratory data in 23 patients with cirrhosis and hepatic coma* *

| Group | Patient | Age and sex | Diagnosis | Biopsy or autopsy | Physical findings† | Mental‡ state | Tremor§ | Comment | Serum total bilirubin | Serum BSP | NPN or B.U.N. | Blood $NH_4$-N (*micrograms per 100 ml.*)‖ Antecubital (A) or femoral (F) vein | Artery | Jugular bulb | A-V diff. extremities | A-V diff. brain |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *mg./100 ml.* | % | *mg./100 ml.* | | | | | |
| Without hemorrhage | 1¶ | 57 M | Cirrhosis | Yes | Icterus, angiomata, ascites | 2° I.C. | 0 | No G.I. bleeding for 24 hours | | 39 | 23 NPN | | 139 | 251 | | −112 |
| | 2 | 62 M | Cirrhosis | Yes | Angiomata, ascites | 1° I.C. | 1+ | 11 days after paracentesis; 3 days after Diamox® | 1.0 | 15 | 42 NPN | (F) 89 | 153 | 182 | + 64 | − 29 |
| | 3¶ | 65 F | Cirrhosis | Yes | Ascites, edema | 3° I.C. | 2+ | Spontaneous coma | 23 | | 28 NPN | (A) 67 | 120 | | + 53 | |
| | 4¶ | 47 F | Cirrhosis | Yes | Angiomata, ascites | 3° I.C. | 1+ | Had phenobarbital and compound F | 3.5 | | 42 | (F) 107 | 169 | 193 | + 62 | − 24 |
| | 5¶ | 38 M | Cirrhosis, myocardial infarction | No | Angiomata, ascites | 1° I.C. | 1+ | 9 days after paracentesis | 1.6 | >40 | 17 | (F) 145 | 226 | 246 | + 81 | − 20 |
| | 6 | 52 M | Cirrhosis | No | Ascites | 2° I.C. | 3+ | Had paraldehyde, ACTH and reserpine | 42 | | 14 | (A) 167 | 164 | 186 | − 3 | − 22 |
| | 7 | 69 M | Cirrhosis, carcinoma of pancreas | Yes | | 1° I.C. | | 4 days after operation | 11.9 | | 21 | (F) 31 | 29 | | − 2 | |
| | 8¶ | 57 M | Cirrhosis | Yes | Angiomata, ascites, edema | 1° C. | 0 | Had Thorazine® and paregoric | 1.2 | 64 | 21 | (A) 266 | 271 | 378 | + 5 | −107 |
| | 9 | 53 M | Cirrhosis, hypertension, cardiac failure | No | Edema | 3° I.C. | 3+ | Received Diamox® | 2.0 | | 34 | (A) 28 | 38 | 47 | + 10 | − 9 |
| | 10¶ | 49 M | Cirrhosis | Yes | Splenomegaly, ascites, edema | 3° C. | 0 | Shock 24 hours previously | 3.2 | >20 | 36 | (F) 265 | 297 | 238 | + 32 | + 59 |
| | 11 | 65 M | Cirrhosis | Yes | Angiomata, ascites, edema | 1° I.C. | 1+ | 150 Gm. protein diet previous 4 days | 4.3 | | 20 | (F) 228 | 207 | 235 | − 21 | − 28 |
| | 12 | 61 M | Cirrhosis | No | Angiomata, ascites | 1° I.C. | 2+ | Had paraldehyde | 5.6 | | 9 | (A) 118 | 119 | | + 1 | |
| | 13 | 38 M | Cardiac cirrhosis, Cor pulmonale | No | Ascites, edema | 1° I.C. | 3+ | 48 hours after Diamox® | 1.0 | >20 | 39 | (A) 112 | 121 | 147 | + 9 | − 26 |
| | | | | | | ? I.C. | 1+ | No further Diamox® | | | 20 | | 113 | 136 | | − 23 |
| | 14 | 51 F | Cirrhosis | Yes | Splenomegaly, edema | 2° I.C. | 2+ | No G.I. bleeding for 24 hours | 1.8 | 51 | 20 | (A) 155 | 156 | 133 | + 1 | + 23 |
| With hemorrhage | 13 | | | | | 1° I.C. | 1+ | G.I. bleeding | | | 41 | (F) 51 | 112 | 102 | + 61 | + 10 |
| | 14 | | | | | 1° I.C. | 3+ | Epistaxis | | | 20 | (A) 142 | 185 | 141 | + 43 | + 44 |
| | 15 | 64 M | Cirrhosis, cholelithiasis | No | Angiomata, splenomegaly, edema | 2° I.C. | 2+ | G.I. bleeding | 1.2 | 24 | 36 NPN | (A) 62 | 197 | | +135 | |
| | 16¶ | 63 M | Cirrhosis | Yes | Ascites, edema | 1° I.C. | ± | G.I. bleeding | 15.2 | 52 | 45 NPN | (F) 71 | 135 | | + 64 | |
| | | | | | | 1° I.C. | 1+ | | | | 45 NPN | (A) 70 | 116 | | + 46 | |
| | | | | | | 1° C. | 0 | | | | 28 NPN | | 203 | 276 | | − 73 |
| | | | | | | 2° C. | 0 | | | | 40 NPN | | 283 | 275 | | + 8 |
| | | | | | | 1° C. | 0 | Epistaxis | | | | (A) 120 | 185 | 145 | + 65 | + 40 |
| | 17 | 64 M | Cirrhosis, peptic ulcer | Yes | Angiomata, edema | 2° I.C. | 2+ | G.I. bleeding | 4.4 | | | (A) 78 | 127 | 87 | + 49 | + 40 |
| | 18 | 78 M | Cirrhosis | No | Angiomata, ascites, edema | 3° I.C. | 2+ | G.I. bleeding | 2.0 | | 37 | (F) 150 | 227 | 195 | + 77 | + 32 |
| | | | | | | 1° I.C. | 1+ | | | | 37 | (F) 83 | 214 | 125 | +131 | + 89 |
| | | | | | | 3° I.C. | 3+ | | 3.2 | 43 | 34 | (A) 404 | 625 | 477 | +221 | +148 |
| | 19 | 61 M | Cirrhosis | No | Angiomata, splenomegaly, ascites | 1° C. | 1+ | G.I. bleeding | 3.5 | 43 | 39 | (A) 109 | 175 | 152 | + 66 | + 23 |
| | 20 | 42 M | Cirrhosis | No | Angiomata, ascites | 2° C. | 2+ | G.I. bleeding | 5.1 | | 17 | (F) 112 | 205 | 172 | + 93 | + 33 |
| | 21¶ | 54 M | Cirrhosis | No | Angiomata, ascites, edema | 3° I.C. | 3+ | G.I. bleeding | 7.4 | 72 | 22 | (A) 211 | 337 | 266 | +126 | + 71 |
| | 22¶ | 64 M | Cirrhosis, hepatoma | Yes | Angiomata, ascites, edema | 1° I.C. | 1+ | G.I. bleeding | 4.9 | | 70 | (F) 87 | 168 | 115 | + 81 | + 53 |
| | 23 | 47 M | Cirrhosis | Yes | Angiomata, ascites, edema | 1° I.C. | 2+ | G.I. bleeding | 6.2 | | | (F) 154 | 242 | 185 | + 88 | + 57 |

* Includes patients with either impending hepatic coma or coma. Patient 8 was the only one receiving an antibiotic (penicillin).
† The liver was palpable except in Patients 2, 3, 5, 11, 15, 20, 21 and 23.
‡ I.C., impending hepatic coma; C, hepatic coma. Both were graded from first to fourth degree (22).
§ "Flapping" tremor graded from 1+ to 4+ (22).
‖ Blood $NH_4$-N determinations are presented separately when they were done on different days in the same patient.
¶ Expired during hospitalization.

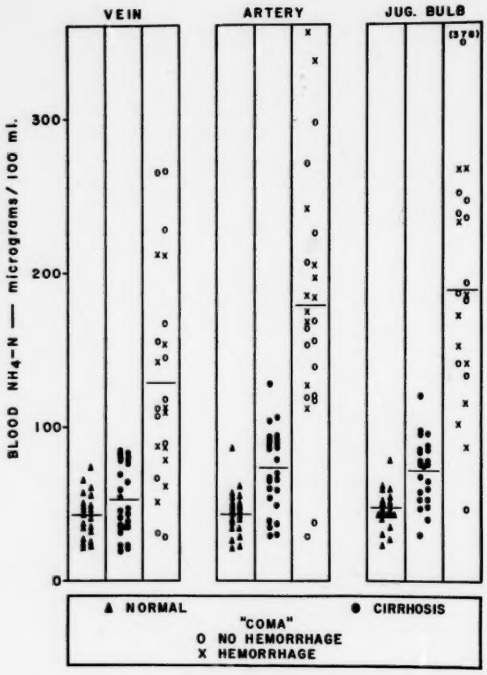



FIG. 1. BLOOD AMMONIUM CONCENTRATIONS

The plotted values represent the concentrations shown in Tables I, II and III. The horizontal line for each group of plotted values indicates the mean concentration for the group.

are charted in Figure 1. Ammonium-nitrogen concentrations in antecubital venous blood of the alert patients with cirrhosis were not significantly higher than those of the group without liver disease ($p = 0.06$). However, $NH_4$–N concentrations measured in arterial or jugular bulb venous blood were significantly higher in the alert patients with cirrhosis than in those without liver disease ($p < 0.01$).

Ammonium-nitrogen concentrations in blood drawn from each site were highest in the "coma" group ($p < 0.01$) but the mean concentrations obtained in patients with and without hemorrhage were not significantly different from each other. Arterial and jugular bulb blood $NH_4$–N values overlapped less between cirrhosis and "coma" groups than did values found in venous blood obtained from the extremities. Although both arterial and jugular bulb blood $NH_4$–N concentrations correlated better with the above clinical classification than did those in extremity venous blood, concentrations in blood from all three sites correlated poorly with the depth of impending hepatic coma or coma (Table III).

*Arteriovenous ammonium-nitrogen differences for the extremities*

Arteriovenous blood $NH_4$–N concentration differences were calculated for individual patients

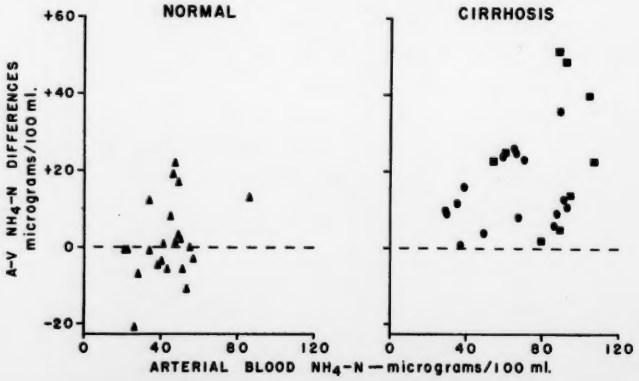



FIG. 2. EXTREMITY A–V AMMONIUM DIFFERENCES

The plotted values are derived from the data in Tables I and II. A positive arteriovenous difference indicates an uptake of $NH_4$–N; a negative difference indicates a release.

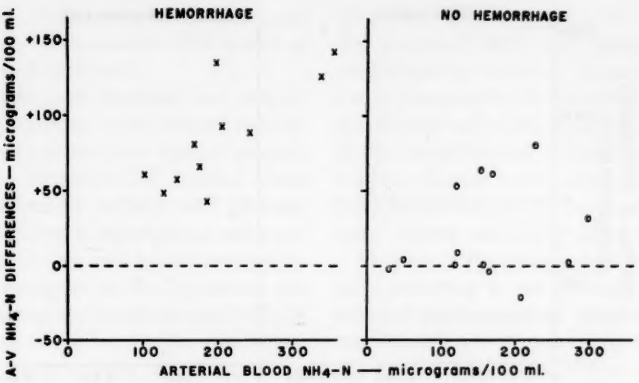



FIG. 3. EXTREMITY A–V AMMONIUM DIFFERENCES IN HEPATIC COMA

The plotted values are derived from data in Table III. Blood $NH_4$–N concentrations and arteriovenous differences determined on separate days in an individual were averaged. A positive arteriovenous difference indicates an uptake of $NH_4$–N; a negative difference indicates a release.

(Tables I, II and III). Each arteriovenous $NH_4$–N difference for the extremities was plotted as a function of its arterial blood $NH_4$–N concentration. In patients without liver disease, individual arteriovenous $NH_4$–N differences were within a range of plus 22 to minus 21 micrograms per 100 ml. of blood. There was neither a significant average uptake nor release of $NH_4$–N per given volume of blood circulating through the extremities. Moreover, there was no correlation between individual arteriovenous blood $NH_4$–N concentration differences and their respective arterial blood $NH_4$–N concentrations (Table I, Figure 2).

In contrast to the group without liver disease, alert patients with cirrhosis demonstrated positive arteriovenous $NH_4$–N differences (uptakes) in their extremities (Table II and Figure 2). When arteriovenous $NH_4$–N differences obtained for the upper extremities in alert patients with cirrhosis were plotted as functions of their respective arterial $NH_4$–N values the resulting correlation was not significant (Figure 2) ($r = 0.373$; $p = 0.07$).

In the 11 "coma" patients with an added nitrogen load in the form of blood in their gastrointestinal tracts the extremities removed more $NH_4$–N from the blood with increasing arterial $NH_4$–N concentrations as expressed by the regression $\hat{Y} = 0.343X$ plus 15.5 (Figure 3) ($r = 0.77$; $p < 0.01$). In contrast, only 4 of 11 patients in the "coma" group without evidence of an added nitrogen load removed ammonium efficiently from a given volume of blood at increased arterial $NH_4$–N concentrations (Figure 3). Thus, at a given arterial blood $NH_4$–N concentration there was a greater removal of $NH_4$–N from a given volume of blood circulating through the extremities of the group of confused or comatose patients with blood in their gastrointestinal tracts than in the group without hemorrhage.

### *Arteriovenous ammonium-nitrogen differences for the brain*

Arteriovenous $NH_4$–N differences for brain, as for the extremities, were plotted as functions of

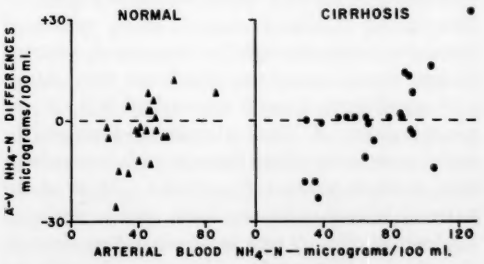



FIG. 4. BRAIN A–V AMMONIUM DIFFERENCES

The values plotted are derived from data in Tables I and II. A positive arteriovenous difference indicates an uptake of $NH_4$–N; a negative difference indicates a release.

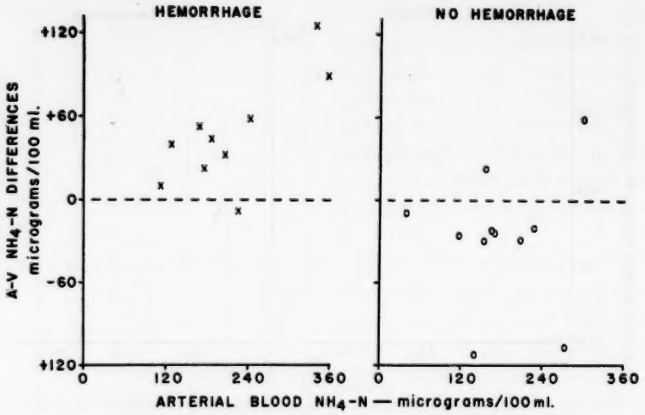



FIG. 5. BRAIN A–V AMMONIUM DIFFERENCES IN HEPATIC COMA

The plotted values are derived from the data in Table III. An average difference is plotted when these differences were determined for the same patient on separate days. A positive arteriovenous difference indicates an uptake of $NH_4$–N; a negative difference indicates a release.

their corresponding arterial blood $NH_4$–N concentrations (Figures 4 and 5).

Arteriovenous differences across the brains of patients without liver disease or of alert patients with cirrhosis were neither consistently positive nor negative, although there was some correlation between this variable and arterial $NH_4$–N concentrations in both groups (Figure 4). In the "coma" group with blood in their gastrointestinal tracts, 9 of the 10 patients showed increasing removal of $NH_4$–N from a given volume of blood circulating through the brain with increasing arterial $NH_4$–N concentrations ($r = 0.74$; $p = 0.01$) as expressed by the regression $\hat{Y} = 0.355X$ minus 28.8 (derived from all values shown in Figure 5). The uptake of $NH_4$–N from a given volume of blood circulating through the brain which occurred in this "coma" group was significant ($p < 0.01$).

A significantly greater removal of $NH_4$–N per given volume of blood circulating through the brain occurred in those patients with hemorrhage than in those without ($p < 0.01$). Most of the patients belonging to the latter group displayed a release of $NH_4$–N into blood circulating through their brains (Figure 5). However, although the average arteriojugular bulb blood $NH_4$–N difference for this group indicated an $NH_4$–N release into the blood, this was not statistically significant ($p = 0.1$).

## DISCUSSION

The removal or release of ammonium from a given volume of blood circulating through the extremities or the brain was obtained by calculating the arteriovenous concentration differences for patients without liver disease, for alert patients with cirrhosis, and for patients with hepatic coma. Although, as discussed below, the interpretation of arteriovenous blood ammonium differences is beset with some difficulties, the $NH_4$–N levels and arteriovenous $NH_4$–N differences in these peripheral sites differed in the groups of patients with cirrhosis when compared with values obtained for patients without hepatic disease. In the patients with liver disease the findings for the extremities differed in some respects from those for the brain.

Arteriovenous $NH_4$–N differences across the extremities ranged from plus 1 to plus 52 mg. per 100 ml. of blood ($NH_4$–N removed) for alert patients with cirrhosis, but were distributed within the range of minus 21 to plus 22 mg. per 100 ml. of blood for patients without liver disease (Figure 2). Arteriovenous $NH_4$–N differences across the brains were not significantly different in the control subjects compared to the values for the alert patients with cirrhosis (Figure 4). Thus there were differences in $NH_4$–N removal for: 1) the extremities of alert patients with cirrhosis

compared to those of the control subjects; and 2) the extremities as contrasted to the brains of the alert patients with cirrhosis.

The alert patients with cirrhosis had arterial $NH_4$–N concentrations that were elevated as compared to the values obtained for control subjects (Figure 1). The elevations of arterial blood $NH_4$–N concentrations in patients with cirrhosis were apparently sufficient to stimulate an enhanced removal of $NH_4$–N by the extremities but not by the brain. Further support for this hypothesis was obtained by comparing the arteriovenous $NH_4$–N differences for brain with those for the extremities in the group of patients in coma with hemorrhage (Figures 3, 5). Positive arteriovenous $NH_4$–N differences (uptakes) for both extremities and brain correlated with the arterial $NH_4$–N concentrations in these patients, but the uptakes of $NH_4$–N occurred at lower arterial concentrations in extremities than they did in brain. In addition, individuals in the coma group who demonstrated positive arteriovenous $NH_4$–N differences for extremities did not always have positive differences for brain (Table III). The explanation for these differences in the metabolism of ammonium in extremities compared to that in brain of the patients with cirrhosis studied is not known. However, ammonium disposal is known to differ in these sites (17), and other dissimilarities might exist as well, *e.g.*, in ammonium transport mechanisms.

The data obtained in comatose patients with hemorrhage suggest that both extremities and brain removed ammonium from a given volume of blood with about equal efficiencies once removal was initiated, since the regression of arteriovenous $NH_4$–N differences upon the arterial concentrations for these two sites was similar (0.343 for extremities and 0.335 for brain). This is at variance with the finding of Bessman, Fazekas, and Bessman (16), that at a given arterial $NH_4$–N concentration the extremities of patients with hepatic coma had twice as large an arteriovenous $NH_4$–N difference as the brain (17).

As predicted by Bessman and Bessman (17), arterial and jugular bulb blood $NH_4$–N concentrations correlated better with the classification of patients into normal, cirrhosis and "coma" groups than did the concentrations in antecubital and femoral venous blood (Figure 1). However, even when arterial and jugular bulb venous blood was analyzed, there was considerable overlap of concentrations between different patient groups and a poor correlation between blood $NH_4$–N concentrations and coma depth in patients belonging to the "coma" group. Actually, an excellent correlation between coma depth and blood $NH_4$–N concentration might not be expected because of the many factors which may affect both variables.

Patients in the coma group were classified clinically according to the presence or absence of associated gastrointestinal bleeding. This classification was made because experience suggests that the prognosis of hepatic coma is better for patients with coma precipitated by a nitrogen load than for patients in whom mental symptoms occur without an associated precipitating factor. Whether or not arteriovenous $NH_4$–N differences increased with increasing arterial $NH_4$–N concentrations varied according to this clinical classification. Arteriovenous blood $NH_4$–N differences for extremities and brain usually increased with increasing arterial $NH_4$–N concentrations in confused or comatose patients with cirrhosis who had blood in their gastrointestinal tracts, *e.g.*, an added nitrogen load. In contrast, $NH_4$–N was not removed efficiently from a given volume of blood circulating through the extremities and brains in most of the patients in the "coma" group who did not have gastrointestinal bleeding. In fact, the brains of some of the latter patients released $NH_4$–N into the circulating blood. The difference in prognosis for the patients with, as contrasted to those without, an added nitrogen load might be related to these observed differences in $NH_4$–N metabolism.

The presence or absence of an added ammonium load (*e.g.*, gastrointestinal bleeding) probably did not *per se* account for the variations in arteriovenous $NH_4$–N differences because high arteriovenous differences were noted for the extremities of some alert patients in the cirrhosis group and for the extremities and brains of others in the "coma" group in whom no evidence of an extra ammonium load was detected. Furthermore, if an increased ammonium load to the systemic circulation were to initiate $NH_4$–N removal in the extremities only by increasing the arterial $NH_4$–N concentration, one would expect large arteriovenous $NH_4$–N differences across the extremities and brains of all patients having markedly ele-

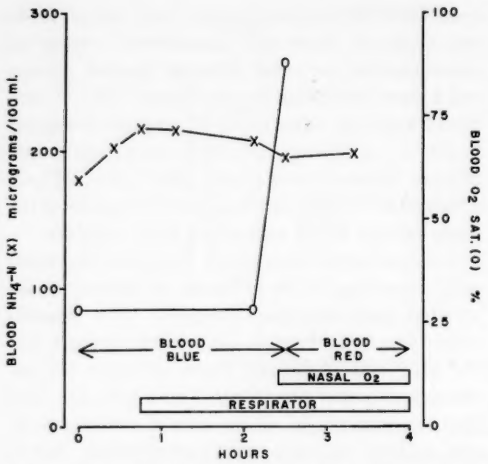



FIG. 6. EFFECT OF ARTERIAL BLOOD $O_2$ SATURATION ON BLOOD AMMONIUM CONCENTRATION

vated arterial $NH_4$–N concentration. This did not always occur (Figures 3, 5). The observed variations in peripheral arteriovenous $NH_4$–N differences might be explained more plausibly by an alteration in the ability of the peripheral intracellular systems either to metabolize ammonium or to initiate ammonium removal at elevated arterial $NH_4$–N concentrations. The negative arteriovenous $NH_4$–N difference across the brains of certain individuals in the coma group might only reflect an inability of their brains to cope with ammonium which is normally produced (33). For example, in animals ammonium concentrations in the brain may increase if fluoroacetate, an agent effective in blocking the tricarboxylic acid cycle, is administered (34). These possibilities require further elucidation.

Aside from difficulties inherent in measuring blood $NH_4$–N,[8] interpretation of arteriovenous $NH_4$–N differences is difficult for several reasons. An arteriovenous difference for a given tissue only determines whether or not there is an overall ammonium removal or release in that tissue; it provides no information concerning the ammonium transport mechanisms involved. The composition of the blood analyzed (arterial as contrasted to venous) might affect the amount of ammonium recovered during analysis. For instance, some investigators suggest that the ammonium concentration in blood is proportional to its oxygen content (35, 36). However, a patient with cor pulmonale studied in this laboratory did not demonstrate significant fluctuations in arterial blood $NH_4$–N concentrations when his arterial oxygen saturation was increased *in vivo* from 28 to 88 per cent (Figure 6). When the quantity of blood circulating through the tissue is not known, arteriovenous differences can provide only semiquantitative estimates of tissue uptake or release. Abnormalities of blood flow do occur in patients with cirrhosis of the liver (37–40), but it seems unlikely that variations of blood flow through the extremities and brain solely accounted for the variations in arteriovenous $NH_4$–N differences noted in the present study. The magnitude of changes in arteriovenous differences observed is probably too great to be explained by changes in blood flow alone. The incidence of clinical findings often associated with altered blood flow through peripheral tissues, *i.e.*, hypotension, palmar erythema, and spider angiomata, was similar for individuals having high or low $NH_4$–N uptakes per given volume of circulating blood. In addition, preliminary studies in this laboratory indicate that the rate of cerebral blood flow, found to be decreased in patients with hepatic coma (39, 40), does not correlate with the arteriovenous $NH_4$–N difference across the brain. In accord with the above reservations, data concerning arteriovenous blood $NH_4$–N differences for peripheral sites have been discussed only in reference to the results obtained for the control group of hospitalized patients without liver disease.

Considering the present findings and assuming that abnormal ammonium metabolism is important in hepatic coma, one might explain this disorder in several ways. The removal of ammonium occurring in the brains of patients with an added nitrogen load suggests that hepatic coma might result because the brain had to metabolize increased quantities of ammonium presented to it by the arterial blood. This process of ammonium disposition might deprive the brain of reactants that are required for other essential metabolic processes. The release of ammonium from the brains of some patients in coma not associated with an

[8] Since ammonium ion is not measured directly by methods devised to date, it is possible that volatile base released from other substances present in blood is also being measured.

added nitrogen load suggests that the confusion or coma associated with hepatic disease might ensue because certain metabolic functions of the brain concerned in ammonium disposal were affected adversely by an abnormally functioning liver. These mechanisms operating simultaneously in a patient with hepatic coma would make the identification and evaluation of each difficult.

## SUMMARY

Ammonium nitrogen was determined in blood obtained practically simultaneously from an extremity vein, an artery and a superior jugular bulb in fasting patients without liver disease, with cirrhosis, and with cirrhosis and either impending hepatic coma or coma.

Arterial and jugular bulb blood ammonium concentrations were least in the group without liver disease, higher in the group with cirrhosis, and highest in the group with impending hepatic coma or coma. Ammonium concentrations in blood obtained from these sites correlated better with the above clinical classification than did those found in extremity venous blood. However, the correlation was not good enough to permit prediction of the clinical state in individual cases.

As judged by arteriovenous blood $NH_4$–N differences alone, there was no significant uptake or release of $NH_4$–N across the extremities and brains of patients without hepatic disease. In patients with liver disease, sufficient increases in arterial blood ammonium concentrations were usually associated with a removal of ammonium from a given volume of blood circulating through these peripheral sites. The extremities of some patients in both the cirrhosis and "coma" groups removed ammonium from a given volume of blood in proportion to the arterial blood ammonium concentrations. A similar removal of ammonium also occurred in the brains of some patients in the "coma" group. This was roughly proportional to the arterial blood ammonium concentration.

Ammonium was not always removed efficiently from a given volume of blood in the extremities and in the brain. Some patients in the cirrhosis group and some in the "coma" group demonstrated a poor ammonium removal for the extremities. Furthermore, not only did the brains of some patients in the latter group remove ammonium poorly but others showed significant releases. In alert patients with cirrhosis the extremities removed ammonium from a given volume of circulating blood when the brain did not.

Confused or comatose patients with hepatic cirrhosis were classified into two groups according to the presence or absence of an added nitrogen load, *i.e.,* blood in the gastrointestinal tract. Although there was overlap between individual values, ammonium removal from given volumes of blood circulating through the extremities and brain was significantly greater in the group with an added nitrogen load than in the group without hemorrhage.

The status of peripheral ammonium removal may be important in the genesis and outcome of hepatic coma.

## ACKNOWLEDGMENTS

These studies were initiated by one of the authors (L.T.W., Jr.) at The Thorndike Memorial Laboratory, Boston City Hospital. The authors wish to express their appreciation to Dr. Charles S. Davidson for his continuing interest in this work.

The authors are also grateful to Dr. Norman P. Shumway for the opportunity to study patients on the Medical Service at the Crile Veterans Administration Hospital, Cleveland, Ohio.

The technical assistance of Miss Alice N. Ballou and Mrs. Helen Grodeck is gratefully acknowledged.

## REFERENCES

1. Gabuzda, G. J., Jr., Phillips, G. B., and Davidson, C. S. Reversible toxic manifestations in patients with cirrhosis of the liver given cation-exchange resins. New Engl. J. Med. 1952, **246**, 124.
2. Phillips, G. B., Schwartz, R., Gabuzda, G. J., Jr., and Davidson, C. S. The syndrome of impending hepatic coma in patients with cirrhosis of the liver given certain nitrogenous substances. New Engl. J. Med. 1952, **247**, 239.
3. Schwartz, R., Phillips, G. B., Seegmiller, J. E., Gabuzda, G. J., Jr., and Davidson, C. S. Dietary protein in the genesis of hepatic coma. New Engl. J. Med. 1954, **251**, 685.
4. McDermott, W. V., Jr., and Adams, R. D. Episodic stupor associated with an Eck fistula in the human with particular reference to the metabolism of ammonia. J. clin. Invest. 1954, **33**, 1.
5. Traeger, H. S., Gabuzda, G. J., Jr., Ballou, A. N., and Davidson, C. S. Blood "ammonia" concentration in liver disease, and liver coma. Metabolism 1954, **3**, 99.
6. Phear, E. A., Sherlock, S., and Summerskill, W. H. J. Blood-ammonium levels in liver disease and "hepatic coma." Lancet 1955, **1**, 836.

7. Bollman, J. L., and Mann, F. C. Studies on the physiology of the liver. XVIII. The effect of removal of the liver on the formation of ammonia. Amer. J. Physiol. 1930, **92**, 92.
8. Krebs, H. A. Urea synthesis *in* The Enzymes, J. B. Summer and K. Myrback, Eds. New York, Academic Press Inc., 1952.
9. Roger, S., and Stahl, J. Correlation entre l'épréuve d'ammoniémie provoquée et l'histopathologie hépatique. C. R. Soc. Biol. (Paris) 1952, **146**, 1786.
10. White, L. P., Phear, E. A., Summerskill, W. H. J., and Sherlock, S. Ammonium tolerance in liver disease: Observations based on catheterization of the hepatic veins. J. clin. Invest. 1955, **34**, 158.
11. Kirk, E. Amino acid and ammonia metabolism in liver diseases. Acta med. scand. 1936, Suppl. **77**, 8.
12. Webster, L. T., Jr., and Davidson, C. S. Cirrhosis of the liver. Impending hepatic coma and increased blood ammonium concentrations during protein hydrolysate infusion. J. Lab. clin. Med. 1957, **50**, 1.
13. Webster, L. T., Jr., and Davidson, C. S. Sources of blood ammonium after feeding protein to patients with hepatic cirrhosis (abstract). J. clin. Invest. 1956, **35**, 742.
14. Fuld, H. Über die diagnostische verwerbarkeit von ammoniakbestimmungen im blut. Klin. Wschr. 1933, **12**, 1364.
15. Young, P. C., Burnside, C. R., Knowles, H. C., Jr., and Schiff, L. The effects of intragastric administration of whole blood on the concentration of blood ammonia in patients with liver disease. J. Lab. clin. Med. 1957, **50**, 11.
16. Bessman, S. P., Fazekas, J. F., and Bessman, A. N. Uptake of ammonia by the brain in hepatic coma. Proc. Soc. exp. Biol. (N. Y.) 1954, **85**, 66.
17. Bessman, S. P., and Bessman, A. N. The cerebral and peripheral uptake of ammonia in liver disease with an hypothesis for the mechanism of hepatic coma. J. clin. Invest. 1955, **34**, 622.
18. Bessman, S. P., and Bradley, J. E. Uptake of ammonia by muscle. Its implications in ammoniagenic coma. New Engl. J. Med. 1955, **253**, 1143.
19. Adams, R. D., and Foley, J. M. Neurological changes in more common types of severe liver disease. Trans. Amer. neurol. Ass. 1949, **74**, 217.
20. Webster, L. T., Jr., and Davidson, C. S. The effect of sodium glutamate on hepatic coma. J. clin. Invest. 1956, **35**, 191.
21. Nelson, R. M., and Seligson, D. Studies of blood ammonia in normal and shock states. Surgery 1953, **34**, 1.
22. Singh, I. D., Barclay, J. A., and Cooke, W. T. Blood-ammonia levels in relation to hepatic coma and the administration of glutamic acid. Lancet 1954, **1**, 1004.
23. McDermott, W. V., Jr., Wareham, J., and Riddell, A. G. Treatment of "hepatic coma" with l-glutamic acid. New Engl. J. Med. 1955, **253**, 1093.
24. Bessman, S. P. The reduction of blood ammonia levels by certain amino acids (abstract). J. clin. Invest. 1956, **35**, 690.
25. Najarian, J. S., and Harper, H. A. A clinical study of the effect of arginine on blood ammonia. Amer. J. Med. 1956, **21**, 832.
26. Fisher, C. J., and Faloon, W. W. Episodic stupor following portacaval shunt. Observations on etiology and therapy. New Engl. J. Med. 1956, **255**, 589.
27. Conway, E. J. Microdiffusion Analysis and Volumetric Error, 3rd ed. New York, D. Van Nostrand Company, Inc., 1950.
28. Gaebler, O. H. Determination of bromsulphalein in normal, turbid, hemolyzed or icteric serums. Amer. J. clin. Path. 1945, **15**, 452.
29. Ducci, H., and Watson, C. J. The quantitative determination of the serum bilirubin with special reference to the prompt-reacting and the chloroform-soluble types. J. Lab clin. Med. 1945, **30**, 293.
30. Myers, V. C. Practical Chemical Analysis of Blood, 2nd ed. St. Louis, C. V. Mosby Company, 1924.
31. Owings, R. H., and Mandel, E. E. Studies in nonprotein nitrogen. I. A convenient method for measuring urea in blood. Proc. Soc. exp. Biol. (N. Y.) 1951, **78**, 363.
32. Snedecor, G. W. Statistical Methods, 4th ed. Ames, Iowa, The Iowa State College Press, 1946.
33. Weil-Malherbe, H. Significance of glutamic acid for the metabolism of nervous tissue. Physiol. Rev. 1950, **30**, 549.
34. Benitez, D., Pscheidt, G. R., and Stone, W. E. Formation of ammonium ion in the cerebrum in fluoroacetate poisoning. Amer. J. Physiol. 1954, **176**, 488.
35. Iber, F. L., and Chalmers, T. C. Biochemical observations on the use of l-glutamic acid in the treatment of hepatic coma. J. clin. Invest. 1957, **36**, 706.
36. Fisher, C. J., Faloon, W. W., Auchincloss, J. H., Eich, R., and Gilbert, R. Alteration in blood ammonia with changing oxygen concentrations (abstract). Gastroenterology 1957, **33**, 269.
37. Kowalski, H. J., Abelmann, W. H., and McNeely, W. F. The cardiac output in patients with cirrhosis of the liver and tense ascites with observations on the effect of paracentesis. J. clin. Invest. 1954, **33**, 768.
38. Abelmann, W. H., and Hutcheson, J. M., Jr. Peripheral blood flow in patients with cirrhosis of the liver at rest and during exercise. Clin. Res. Proc. 1956, **4**, 147.
39. Wechsler, R. L., Crum, W., and Roth, J. L. A. The blood flow and $O_2$ consumption of the human brain in hepatic coma (abstract). Clin. Res. Proc. 1954, **2**, 74.
40. Fazekas, J. F., Ticktin, H. E., Ehrmantraut, W. R., and Alman, R. W. Cerebral metabolism in hepatic insufficiency. Amer. J. Med. 1956, **21**, 843.

# THE BLOOD VOLUMES AND PLASMA PROTEIN DURING LEVARTERENOL–INDUCED HYPERTENSION [1]

By FRANK A. FINNERTY, JR.,[2] JOACHIM H. BUCHHOLZ, AND ROBERT L. GUILLAUDEU

*(From the Georgetown University Medical Division, District of Columbia General Hospital, Washington, D. C.)*

(Submitted for publication August 20, 1957; accepted November 14, 1957)

Studies now in progress in this laboratory suggest that acute reduction of arterial pressure in some hypertensive patients is followed immediately by an increase in plasma volume and a decrease in hematocrit and plasma protein. In order to study this phenomenon further, it seemed worthwhile to perform the reverse experiment, *i.e.*, acute elevation of the arterial pressure.

Levarterenol (Levophed®) was selected as the pressor agent, first because it produces hemodynamic changes resembling those of hypertension, and second because the arterial pressure could be increased significantly in an asymptomatic fashion.

A review of the literature in reference to the volume of blood and its components after administration of pressor substances is confusing. Prior to 1941, as many investigators found an increase in plasma volume as a decrease (1–4). In 1941, Ebert and Stead (5), and in 1942 Kaltreider, Meneely, and Allen (6), independently found hemoconcentration following epinephrine and explained that the previous discrepancies were due to the fact that other investigators were not allowing time for mixing of dye and plasma prior to the experimental period. At that time, no definite conclusions could be drawn regarding the effect of epinephrine on red cell mass since only indirect methods were available. The evidence suggested, however, that a few red cells were added to the circulation following epinephrine.

It was hoped that the simultaneous measurement of plasma volume and red cell mass by direct methods before and during levarterenol infusion would clarify some of the questions regarding the change of the blood volume and its components after pressor substances.

## MATERIALS AND METHODS

Experimental procedures were carried out on 10 patients without obvious cardiovascular disease. Five were female and five were male.

The plasma volume, red cell mass, hematocrit and plasma protein were determined in all patients before and during the hypertensive state produced by levarterenol. The patients were in the fasting state. Following a blank sample, a suspension of the patient's red cells incubated with $Cr^{51}$ and washed three times in standard fashion was injected quantitatively into the antecubital vein (7). Two minutes following this injection, a known amount of T-1824 dye was injected into the same antecubital vein with a different syringe. The precise amount of the dye and isotope delivered was determined by a control *in vitro* injection. Venous samples were drawn from the opposite arm, without the use of a tourniquet, and collected in tubes containing dried heparin at 10, 15, 20 and 25 minutes after the injection of the dye for the determinations of optical density, radioactivity, hematocrit and protein. The optical density of the plasma was measured in a spectrophotometer [3] at a wave length of 620 mμ. No corrections were necessary for hemolysis. The radioactivity of the whole blood samples was determined in a scintillation well counter.[4] The plasma volume and red cell mass were calculated by extrapolating the optical density and radioactivity to the times of injection. The hematocrit was measured in Wintrobe tubes. The plasma protein concentration was determined by the copper sulfate specific gravity method (8).

Immediately following the withdrawal of the 25 minute venous sample, an intravenous infusion of diluted levarterenol was started (4 ml. levarterenol bitartrate diluted in 1,000 ml. five per cent dextrose in water). Since pain and anxiety by themselves might further alter the various parameters measured in these experiments, care was taken to keep the acute drug-induced hypertensive state asymptomatic. If the arterial pressure rise

[1] This investigation was supported by research grants from: The American Heart Association; The Washington Heart Association, Washington, D. C.; The National Heart Institute (H-2509), National Institutes of Health, Bethesda, Md.; Irwin, Neisler & Co., Decatur, Ill.; Ciba Pharmaceuticals, Inc., Summit, N. J.; and Charles Pfizer & Co., Inc., Brooklyn, N. Y.

[2] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

[3] Coleman Instruments, Inc., Maywood, Ill.

[4] Nuclear Instrument and Chemical Corp., Chicago, Ill.

were gradual (*i.e.*, over a 10 to 15 minute period), the mean arterial pressure could be increased by more than 40 per cent without causing untoward symptoms. All arterial pressures were recorded by sphygmomanometer and the mean arterial pressure calculated from the formula (systolic plus diastolic pressure)/2. Once the levarterenol infusion was started, the blood pressure was recorded every minute and the speed of injection regulated to insure an asymptomatic arterial pressure rise. Three additional venous samples were drawn at five minute intervals while the arterial pressure was being acutely increased. When the arterial pressure had stabilized, an additional venous sample was drawn for reference which was immediately followed by a second injection of $Cr^{51}$ labelled red cells and T-1824. A typical experiment is depicted in Figure 1.

## RESULTS

The effect of levarternol on the blood volume and its various components is shown in Table I. An average increase of 56 per cent in mean arterial pressure was associated with an average decrease of 15 per cent in plasma volume ($p < 0.01$), no change in red cell mass, an average increase of 8 per cent in hematocrit ($p < 0.01$), and an average increase of 5 per cent in plasma protein ($p = 0.05$).

The protein pool [plasma volume (T-1824) times protein concentration] decreased after the administration of levarterenol from an average of

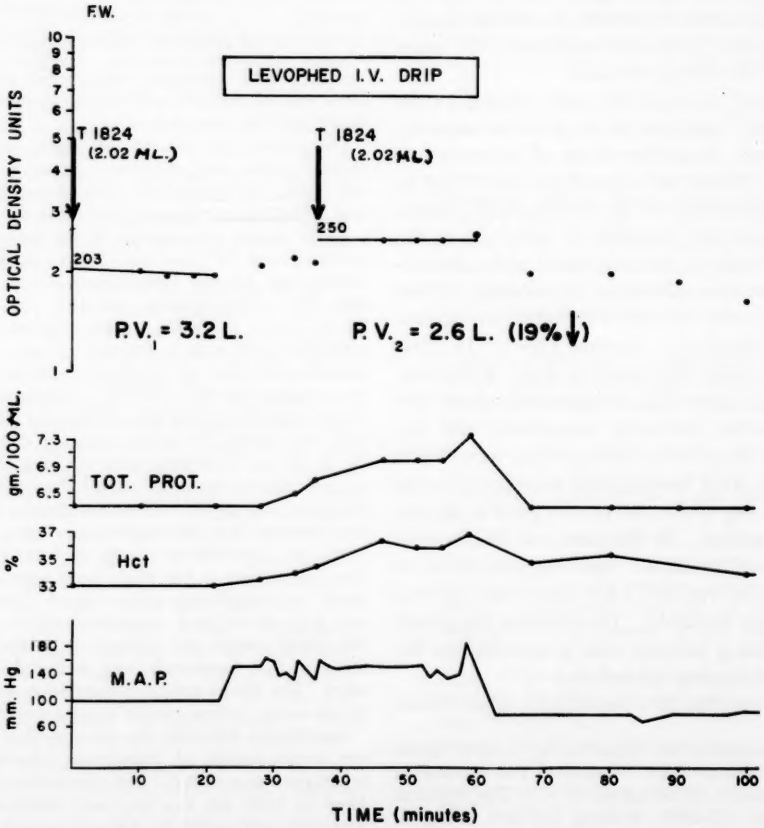



FIG. 1. A TYPICAL EXPERIMENT SHOWING THE EFFECT OF LEVARTERENOL ON THE ARTERIAL PRESSURE, HEMATOCRIT, TOTAL PROTEIN AND PLASMA VOLUME

The red cell mass is not plotted since no change occurred. Note the evidence of hemoconcentration from all parameters and the immediate relationship between changes in arterial pressure and hematocrit, protein and the individual readings of optical density of T-1824.

215 grams to 193 ($p = 0.5$) grams which represents an average loss of 22 grams or 10 per cent of the plasma proteins (Table II).

When the calculation of the protein pool is based on indirect measurements of plasma volume (plasma volume equals (red cell mass/hematocrit) minus red cell mass), levarterenol infusion was accompanied by a decrease in protein pool from an average of 155 grams to 143 grams (12 grams or 8 per cent)[5] ($p = 0.7$). Although the changes in protein pools are not significant ($p = 0.5$, and $p = 0.7$), it should be noted in Table II that all changes in protein concentrations are in the same direction.

## DISCUSSION

Regardless of the parameter studied, the data suggest that the hypertension induced by levarterenol is associated with hemoconcentration. Compared to control determinations, there is an increase in the percentage of packed red cells, the radioactivity per ml. of whole blood, the optical density of the T-1824 dyed plasma and in the concentration of plasma protein.

Since the plasma proteins in these experiments were measured by the copper sulfate specific gravity method, the effect of changes in the nonprotein constituents of the plasma has to be considered. The studies of Bernstein (9) and Lowry and Hunter (10) have shown that even gross changes of nonprotein constituents do not appreciably alter the protein concentration as measured by specific gravity methods (*e.g.*, each 100 mg. per cent increase in glucose causes an increase in the calculated protein of only 0.1 to 0.15 gram per 100 ml.). It was concluded, therefore, that the increase in specific gravity of the serum noted in these experiments during levarterenol represents an increase in plasma protein concentration.

It has been shown that epinephrine can cause an increase in the optical density of the nondyed plasma (11). The concomitant increase in hematocrit, concentration of protein and T-1824 in the plasma (Figure 1) during levarterenol indicates that the increase in the optical density of the dyed plasma mainly represents hemoconcentration. This interpretation is further substantiated by work in progress in this laboratory substituting

[5] Calculation of the protein pool was based on only nine patients.

TABLE I

*Plasma volume, red cell mass, and plasma protein during levarterenol-induced hypertension*

| Name | Sex | Age | M.A.P.* mm. Hg | | | P.V.† liters | | | P.V. ml./Kg. | | R.C.M.‡ liters | | | Hct.§ per cent | | | P.P.‖ grams/100 ml. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C¶ | E¶ | % increase | C | E | % decrease | C | E | C | E | % increase | C | E | % increase | C | E | % increase |
| | | *yrs.* | | | | | | | | | | | | | | | | | |
| W. D. | M | 58 | 95 | 150 | 58 | 2.8 | 2.6 | 7 | 47 | 43 | 1.7 | 1.7 | 0 | 41 | 44 | 7 | 7.0 | 7.2 | 3 |
| T. W. | M | 36 | 90 | 185 | 89 | 2.1 | 1.6 | 24 | 37 | 28 | 1.3 | 1.3 | 0 | 47 | 49 | 6 | 7.5 | 8.0 | 7 |
| M. K. | F | 66 | 100 | 140 | 40 | 1.5 | 1.2 | 20 | 27 | 20 | 0.9 | 0.9 | 0 | 43 | 45 | 5 | | | |
| E. R. | M | 44 | 80 | 135 | 69 | 3.0 | 2.8 | 7 | 58 | 55 | 1.0 | 1.0 | 0 | 37 | 41 | 11 | 4.5 | 4.7 | 4 |
| L. F. | F | 15 | 90 | 145 | 61 | 2.7 | 2.3 | 15 | 45 | 38 | 1.3 | 1.3 | 0 | 41 | 45 | 10 | 5.9 | 6.0 | 2 |
| I. K. | M | 44 | 100 | 140 | 40 | 3.5 | 3.2 | 9 | 43 | 39 | 1.3 | 1.3 | 0 | 37 | 40 | 8 | 7.7 | 8.1 | 5 |
| L. R. | F | 29 | 115 | 165 | 44 | 5.1 | 3.8 | 26 | 45 | 34 | 2.5 | 2.5 | 0 | 40 | 43 | 8 | 6.1 | 6.6 | 8 |
| J. E. | M | 34 | 105 | 160 | 52 | 4.5 | 4.1 | 9 | 60 | 54 | 1.7 | 1.7 | 0 | 35 | 38 | 9 | 6.7 | 7.0 | 5 |
| M. N. | F | 19 | 95 | 135 | 42 | 3.1 | 2.4 | 23 | 25 | 19 | 1.3 | 1.3 | 0 | 35 | 39 | 11 | 7.0 | 7.8 | 11 |
| G. B. | F | 32 | 85 | 140 | 65 | 2.5 | 2.2 | 12 | 51 | 46 | 0.9 | 0.9 | 0 | 33 | 36 | 9 | 7.3 | 7.6 | 4 |
| Average | | | 96 | 150 | 56 | 3.1 | 2.6 | 15 | 44 | 38 | 1.4 | 1.4 | 0 | 39 | 42 | 8 | 6.6 | 7.0 | 5 |
| Standard deviation | | | ±10 | ±16 | ±16 | ±0.9 | ±0.9 | ±8 | ±11.5 | ±12.6 | ±.48 | ±.48 | ±0 | ±4 | ±4 | ±2 | ±1.0 | ±1.1 | ±3 |
| p** | | | 0.02 | | | <0.01 | | | <0.01 | | | | | <0.01 | | | 0.05 | | |

* Mean Arterial Pressure.
† Plasma Volume.
‡ Red Cell Mass.
§ Hematocrit.
‖ Plasma Protein.
¶ C, Control; E, Experimental.
** Significance of mean changes.

TABLE II

*Protein pool during levarterenol-induced hypertension*

| | P.V. (T-1824) liters | | P.P. grams/100 ml. | | Protein pool* grams | | | | P.V. ($Cr^{51}$) liters | | P.P. grams/100 ml. | | Protein pool* grams | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Experimental decrease | | | | | | | | | Experimental decrease | |
| | C | E | C | E | C | E | Decrease | Estimated decreasef | C | E | C | E | C | E | Decrease | Estimated decrease | Estimated decrease | Estimated decrease |
| W. D. | 2.8 | 2.6 | 7.0 | 7.2 | 196 | 187 | 9 | 14 | 2.5 | 2.2 | 7.0 | 7.2 | 175 | 158 | 17 | 21 | 0.81 | |
| T. W. | 2.1 | 1.6 | 7.5 | 8.0 | 158 | 128 | 30 | 38 | 1.5 | 1.4 | 7.5 | 8.0 | 113 | 112 | 1 | 8 | 0.13 | |
| E. R. | 3.0 | 2.8 | 4.5 | 4.7 | 135 | 132 | 3 | 9 | 1.7 | 1.4 | 4.3 | 4.7 | 77 | 66 | 11 | 14 | 0.79 | |
| L. F. | 2.7 | 2.3 | 5.9 | 6.0 | 159 | 138 | 21 | 24 | 1.9 | 1.6 | 5.9 | 6.0 | 112 | 96 | 16 | 18 | 0.89 | |
| I. K. | 3.5 | 3.2 | 7.7 | 8.1 | 270 | 259 | 11 | 23 | 2.2 | 2.0 | 7.7 | 8.1 | 169 | 162 | 7 | 15 | 0.47 | |
| L. R. | 5.1 | 3.8 | 6.1 | 6.6 | 311 | 251 | 60 | 79 | 3.8 | 3.3 | 6.1 | 6.6 | 232 | 218 | 14 | 31 | 0.45 | |
| J. E. | 4.5 | 4.1 | 6.7 | 7.0 | 302 | 287 | 15 | 27 | 3.2 | 2.8 | 6.7 | 7.0 | 214 | 196 | 18 | 27 | 0.67 | |
| M. N. | 3.1 | 2.4 | 7.0 | 7.8 | 217 | 187 | 30 | 49 | 2.4 | 2.0 | 7.0 | 7.8 | 168 | 156 | 12 | 28 | 0.43 | |
| G. B. | 2.5 | 2.2 | 7.3 | 7.6 | 183 | 167 | 16 | 22 | 1.8 | 1.6 | 7.3 | 7.6 | 131 | 122 | 9 | 15 | 0.60 | |
| Average | 3.3 | 2.7 | 6.6 | 7.0 | 215 | 193 | 22 | 32 | 2.3 | 2.0 | 6.6 | 7.0 | 155 | 143 | 12 | 20 | 0.58 | |
| St. dev. | ±1.0 | ±0.8 | ±1.0 | ±1.1 | ±65 | ±59 | ±17 | ±21 | ±0.8 | ±0.7 | ±1.0 | ±1.1 | ±51 | ±48 | ±5 | ±7 | ±0.24 | |
| p | <0.01 | | 0.05 | | 0.5 | | | | 0.4 | | 0.05 | | 0.7 | | | | | |

* Protein pool equals plasma volume times protein concentration.

† Estimated decrease equals decrease of plasma volume times protein concentration.

$I^{131}$ for T-1824 which shows an increase in the radioactivity of the plasma during levarterenol infusion (12).

The data presented here are in accord with those of Ebert and Stead (5) and Kaltreider, Meneely, and Allen (6), who demonstrated hemoconcentration during epinephrine-induced hypertension. Our data point out, however, that red cells are not added to the circulation as these investigators had postulated. The increased radioactivity of the whole blood is completely accounted for by the increased hematocrit.

The close relationship between the change in arterial pressure, hematocrit and plasma protein concentration is evident in Figure 1. It is also clear that these changes are quickly reversed when the levarteranol is discontinued.

Increased hydrostatic capillary pressure seems the most likely explanation for the decrease of plasma volume caused by levarterenol. Goldenberg, Pines, Baldwin, Greene, and Roh (13) and McDowall (14) have shown that levarterenol causes an increase in venous pressure and by inference an increase in hydrostatic capillary pressure. In addition, the generalized elevation of arterial pressure would also tend to increase the hydrostatic pressure. The absence of change in the red cell mass eliminates the possibility of a portion of the circulation being trapped. It would seem that the sequestration of plasma in temporarily closed capillaries (or capillaries with a very slow circulation) is excluded as the sole explanation for hemoconcentration, since an increase in the optical density of the plasma occurs before a second injection of T-1824.

The protein pool is larger when the T-1824 value for the plasma volume is used for calculation than when the indirect chromium method is used. The most likely explanation for this discrepancy lies in the fact that the space measured by the T-1824 is greater than the one simultaneously measured with $Cr^{51}$.

It is interesting to note that although the magnitude of the protein pool varies with the method used to measure the plasma volume, the decrease during levarterenol infusion is fairly proportional (10 and 8 per cent) (Table II). This proportional decrease is explained by the fact that both the volume of distribution of T-1824 and the de-

gree of hemoconcentration during levarterenol are greater than when measured by chromium.

The average ratio of protein loss as found in the experiment to the protein loss when calculated from the decrease in plasma volume was 0.64 (T-1824), compared to 0.58 ($Cr^{51}$) (Table II). These data would seem to indicate that the fluid lost from the vascular compartment during levarterenol-induced hemoconcentration contains an average of 64 per cent (T-1824) or 58 per cent ($Cr^{51}$) of the protein concentration of the plasma.

## CONCLUSIONS

1. The hypertensive state produced by levarterenol is associated with an increase in hematocrit and plasma protein, a decrease in plasma volume, and no change in the red cell mass.

2. The fluid lost during hemoconcentration contains less protein than the plasma.

## REFERENCES


1. Wollheim, E. Die zirkulierende Blutmenge und ihre Bedeutung für Kompensation und Dekompensation des Kreislaufs. Z. klin. Med. 1931, **116**, 269.
2. Brandt, F. Die Abhängigkeit des Venendruckes von der Grösse der zirkulierenden Blutmenge, zugleich ein Beitrag zur Frage seiner klinischen Bedeutung. Z. klin. Med. 1931, **116**, 398.
3. Levin, E. La accion de la adrenalina sobre el volumen de la sangre circulante. Rev. méd. Rosario 1935, **25**, 255.
4. Hitzenberger, K., and Tuchfeld, F. Über den Einfluss des Adrenalins auf die zirkulierende Blutmenge. Klin. Wschr. 1929, **8**, 1208.
5. Ebert, R. V., and Stead, E. A., Jr. Demonstration that in normal man no reserves of blood are mobilized by exercise, epinephrine, and hemorrhage. Amer. J. med. Sci. 1941, **201**, 655.
6. Kaltreider, N. L., Meneely, G. R., and Allen, J. R. The effect of epinephrine on the volume of the blood. J. clin. Invest. 1942, **21**, 339.
7. Sterling, K., and Gray, S. J. Determination of the circulating red cell volume in man by radioactive chromium. J. clin. Invest. 1950, **29**, 1614.
8. Phillips, R. A., Van Slyke, D. D., Emerson, K., Hamilton, P. B., and Archibald, R. M. The copper sulfate method for measuring specific gravities of whole blood and plasma. Bull. U. S. Army med. Dep. 1943, no. 71, 66.
9. Bernstein, R. E. The relation between plasma specific gravity or water content and plasma protein concentration in normal and abnormal bloods. S. Afr. J. med. Sci. 1954, **19**, 131.
10. Lowry, O. H., and Hunter, T. H. The determination of serum protein concentration with a gradient tube. J. biol. Chem. 1945, **159**, 465.
11. Ebert, R. V., and Stead, E. A., Jr. An error in measuring changes in plasma volume after exercise. Proc. Soc. exp. Biol. (N.Y.) 1941, **46**, 139.
12. Finnerty, F. A., Jr., Buchholz, J. H., and Tuckman, J. Unpublished data.
13. Goldenberg, M., Pines, K. L., Baldwin, E. DeF., Greene, D. G., and Roh, C. E. The hemodynamic response of man to norepinephrine and epinephrine and its relation to the problem of hypertension. Amer. J. Med. 1948, **5**, 792.
14. McDowall, R. J. S., Ed. Ciba Foundation Symposium on Visceral Circulation. Boston, Little Brown and Co., 1953.

# MAGNESIUM CONSERVATION IN THE HUMAN BEING ON A LOW MAGNESIUM DIET [1]

By BENJAMIN A. BARNES, OLIVER COPE, AND TIMOTHY HARRISON [2]

*(From the Department of Surgery, Harvard Medical School, and the Massachusetts General Hospital, Boston, Mass.)*

(Submitted for publication July 30, 1957; accepted October 31, 1957)

In recent years the metabolism of various minerals has been studied in humans by providing diets that are completely or relatively deficient in a particular mineral yet satisfactory in all other nutritional requirements. Many observations (1–6) record the application of this technique to problems in calcium metabolism, and more recently sodium (7, 8) and potassium (9–19) have been investigated by similar methods. The earliest animal experiments to our knowledge in magnesium metabolism, using a diet designed to create a specific deficiency of this element, were those reported by Osborne and Mendel (20). However, these authors only achieved a minimum concentration of 0.012 per cent magnesium in their diets for rats. The first comparable studies in humans were reported by Fitzgerald and Fourman (21), who employed a diet low in magnesium and also a cation exchange resin to produce a depletion via the gastrointestinal tract.

The work presented in this report does not attempt to create a magnesium deficiency, but rather determines the efficiency of the body in conserving magnesium while on a magnesium-free diet. The interpretation of previous magnesium balance studies (4, 22–26) has been obscured by large intakes which make it difficult, if not impossible, to evaluate small positive or negative balances. These and other reports (27, 28) are not in agreement in defining the requirements of healthy adults, and consequently, it is necessary to establish the efficiency of the conserving mechanisms present in the body as a clue to the daily magnesium requirements.

## METHODS

*Subjects.* These were four healthy females ranging in age from 21 to 25 years. Their medical history was not significant, and they were in a state of robust health at the time of the study. Participation was voluntary, and their personal association with the laboratory and their interest in the investigation gave us complete confidence in the faithful observance of the dietary restrictions and in the meticulous collection of the specimens. The four subjects were ambulatory throughout the study and continued to be active at secretarial or technical work in a laboratory. Vigorous exercise was prohibited although exercise such as walking was not restricted.

In Case 1 the 18 day balance study was started on the tenth day of her normal 29 day menstrual cycle, and in Case 2 the study was started on the first day of her normal 28 day menstrual cycle.

*Diet.* A magnesium-free and calcium-low diet was obtained by taking a patented powdered milk product [3] and reconstituting it with water to the following composition: protein, 1.8 per cent; lactose, 14 per cent; butterfat, 6.1 per cent; and approximately 1.2 calories per ml. The low protein content and high caloric value of the reconstituted liquid are noteworthy in contrast to whole milk. This liquid was processed by percolation through a cation exchange resin column prepared in the sodium and potassium cycle in the ratio of two to three, to approximate the proportion of these elements in whole milk. Iron, manganese, iodine, zinc, copper and cobalt were added in trace amounts to the column effluent. The nitrogen content was unaltered by the resin, the calcium content was reduced to 0.2 mM per liter, and the final concentrations of the potassium and sodium were adjusted to 85 and 74 mEq. per liter, respectively. Analysis of the processed dairy product by flame spectrophotometry, by ammonium phosphate precipitation, and by the titan yellow dye technique indicated that the magnesium content is in the order of one part per million (aproximately 0.08 mEq. per liter) or less. Since each of these methods is not entirely satisfactory for the detection of magnesium in the presence of other ions, we have arbitrarily set a value of 0.12 mEq. per liter as the greatest concentration to be expected and as the magnesium content of the liquid diet. In all probability the magnesium content is far less, an impression confirmed by the complete removal of added radioactive magnesium ($Mg^{28}$) in the upper half of the cation exchange resin column. The details of the production, of the chemical analyses of the diet, and of the biological assay in rats have been reported elsewhere (29).

[1] The work in this paper was supported in part by the United States Public Health Service and by the Office of Naval Research, NR 114-198.

[2] Public Health Service Fellow.

[3] Pream, manufactured by the M & N Dietetic Laboratories, Inc., Columbus, Ohio.

Prior to and following the consumption of this liquid diet the subjects were allowed to take *ad lib.* a diet of their choice, provided that the composition of the diet could be determined from standard food tables (30–32). The monotony of the liquid diet was relieved by a limited supply of additional foods that were virtually free of magnesium. The minor contribution of these to the intake was determined from the food tables.

*Weight.* The subjects were weighed frequently throughout the study in the morning upon arising prior to consuming any food and after voiding. These weights were obtained with the subjects nude or clad in underclothing only.

*Balance method.* During the first and last metabolic periods in the program of balance study detailed below, the intake of nitrogen and minerals was obtained from standard food tables (30–32) employing estimates made by an experienced dietitian of the weight of the food servings. During the middle metabolic periods, in which the liquid diet was consumed, less than 5 per cent of the intake came from additional foods of negligible magnesium content; consequently, the intake of nitrogen and minerals was precisely known because of the direct analysis of the liquid diet. The balance method employed relied on food tables for obtaining the intake only during the initial and final metabolic periods, and such errors as this may introduce would not affect the data from the periods of liquid diet consumption.

The output in the urine was determined on a daily basis, and the stool output was determined from the analy-

TABLE I

*Daily nitrogen and mineral balances of Case 1*

*(N expressed in Gm.; Na, K, Mg in mEq.)*

| Day | Period | N | Na | K | Mg | N | Na | K | Mg | N | Na | K | Mg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Urine | | | | Stool | | | | Total output | | | |
| 1 | | 13.5 | 152 | 126 | 6.89 | 1.78 | 3.1 | 12.6 | 22.6 | 15.3 | 155 | 139 | 29.5 |
| 2 | I | 9.5 | 147 | 101 | 9.84 | 1.78 | 3.1 | 12.6 | 22.6 | 11.3 | 150 | 113 | 32.4 |
| 3 | | 8.4 | 111 | 51 | 6.00 | 1.78 | 3.1 | 12.6 | 22.6 | 10.2 | 114 | 63 | 28.6 |
| 4 | | 8.6 | 134 | 94 | 6.12 | 1.78 | 3.1 | 12.6 | 22.6 | 10.4 | 137 | 107 | 28.7 |
| 5 | II | 5.0 | 50 | 12 | 3.01 | 1.45 | 20.5 | 15.2 | 11.4 | 6.5 | 71 | 27 | 14.4 |
| 6 | | 9.9 | 109 | 87 | 2.91 | 1.45 | 20.5 | 15.2 | 11.4 | 11.4 | 129 | 102 | 14.3 |
| 7 | | 4.6 | 42 | 86 | 1.00 | 0.79 | 13.8 | 9.7 | 0.28 | 5.4 | 55 | 96 | 1.28 |
| 8 | III | 5.4 | 94 | 89 | 0.53 | 0.79 | 13.8 | 9.7 | 0.28 | 6.2 | 107 | 98 | 0.81 |
| 9 | | 3.7 | 117 | 40 | 0.77 | 0.79 | 13.8 | 9.7 | 0.28 | 4.5 | 130 | 50 | 1.05 |
| 10 | | 6.5 | 173 | 100 | 0.42 | 0.79 | 13.8 | 9.7 | 0.28 | 7.3 | 187 | 110 | 0.70 |
| 11 | | 4.6 | 78 | 60 | 0.55 | 0.66 | 11.9 | 9.1 | 0.02 | 5.3 | 90 | 69 | 0.57 |
| 12 | IV | 5.2 | 110 | 80 | 0.40 | 0.66 | 11.9 | 9.1 | 0.02 | 5.9 | 122 | 89 | 0.42 |
| 13 | | 5.5 | 101 | 78 | 0.50 | 0.66 | 11.9 | 9.1 | 0.02 | 6.2 | 113 | 87 | 0.52 |
| 14 | | 5.2 | 86 | 86 | 0.70 | 0.66 | 11.9 | 9.1 | 0.02 | 5.9 | 98 | 95 | 0.72 |
| 15 | | 9.6 | 129 | 86 | 5.01 | 0.97 | 0.96 | 15.4 | 17.2 | 10.6 | 129 | 101 | 22.2 |
| 16 | V | 14.4 | 119 | 95 | 6.32 | 0.97 | 0.96 | 15.4 | 17.2 | 15.4 | 120 | 111 | 23.5 |
| 17 | | 7.4 | 87 | 66 | 4.91 | 0.97 | 0.96 | 15.4 | 17.2 | 8.4 | 88 | 81 | 22.1 |
| 18 | | 8.9 | 100 | 48 | 7.83 | 0.97 | 0.96 | 15.4 | 17.2 | 9.9 | 101 | 63 | 25.0 |
| | | Intake | | | | Balance | | | | | | | |
| 1 | | 14.5 | 158 | 99 | 26 | −0.8 | + 3 | −40 | − 4.0 | | | | |
| 2 | I | 11.8 | 197 | 83 | 25 | +0.5 | +47 | −30 | − 7.0 | | | | |
| 3 | | 7.1 | 205 | 58 | 17 | −3.1 | +91 | − 5 | −12.0 | | | | |
| 4 | | 16.5 | 140 | 124 | 50 | +6.1 | + 3 | +17 | +21.0 | | | | |
| 5 | II | 3.9 | 102 | 81 | 0.6 | −2.6 | +31 | +54 | −14.0 | | | | |
| 6 | | 4.9 | 125 | 99 | 0.7 | −6.5 | − 4 | − 3 | −14.0 | | | | |
| 7 | | 4.9 | 125 | 100 | 0.9 | −2.4 | +70 | + 4 | − 0.4 | | | | |
| 8 | III | 5.3 | 125 | 98 | 1.1 | −0.9 | +18 | 0 | + 0.3 | | | | |
| 9 | | 4.1 | 101 | 79 | 0.6 | −0.4 | −29 | +29 | − 0.5 | | | | |
| 10 | | 4.7 | 117 | 91 | 0.7 | −2.6 | −70 | −19 | 0.0 | | | | |
| 11 | | 4.1 | 102 | 84 | 0.7 | −1.2 | +12 | +15 | + 0.1 | | | | |
| 12 | IV | 4.3 | 109 | 88 | 0.8 | −1.6 | −13 | − 1 | + 0.4 | | | | |
| 13 | | 4.8 | 125 | 99 | 0.7 | −1.4 | +12 | +12 | + 0.2 | | | | |
| 14 | | 4.8 | 125 | 99 | 0.7 | −1.1 | +27 | + 4 | 0.0 | | | | |
| 15 | | 17.7 | 173 | 111 | 29 | +7.1 | +44 | +10 | + 7.0 | | | | |
| 16 | V | 19.4 | 197 | 90 | 31 | +4.0 | +77 | −21 | + 7.0 | | | | |
| 17 | | 15.6 | 187 | 94 | 26 | +7.2 | +99 | +13 | + 4.0 | | | | |
| 18 | | 10.1 | 166 | 56 | 16 | +0.2 | +65 | − 7 | − 9.0 | | | | |

TABLE II

*Daily nitrogen and mineral balances of Case 2*

*(N expressed in Gm.; Na, K, Mg in mEq.)*

| Day | Period | N | Na | K | Mg | N | Na | K | Mg | N | Na | K | Mg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Urine | | | | Stool | | | | Total output | | | |
| 1 | | 10.1 | 107 | 56 | 6.07 | 1.60 | 3.4 | 13.8 | 13.2 | 11.7 | 110 | 70 | 19.3 |
| 2 | I | 10.7 | 137 | 62 | 12.78 | 1.60 | 3.4 | 13.8 | 13.2 | 12.3 | 140 | 76 | 26.0 |
| 3 | | 9.9 | 196 | 76 | 8.56 | 1.60 | 3.4 | 13.8 | 13.2 | 11.5 | 199 | 90 | 21.8 |
| 4 | | 10.3 | 214 | 68 | 9.99 | 1.60 | 3.4 | 13.8 | 13.2 | 11.9 | 217 | 82 | 23.2 |
| 5 | II | 8.9 | 109 | 58 | 5.25 | 2.10 | 21.5 | 19.0 | 10.6 | 11.0 | 131 | 77 | 15.9 |
| 6 | | 7.3 | 131 | 54 | 1.18 | 2.10 | 21.5 | 19.0 | 10.6 | 9.4 | 153 | 73 | 11.8 |
| 7 | | 5.9 | 170 | 132 | 1.13 | 0.56 | 4.6 | 6.0 | 0.04 | 6.5 | 175 | 138 | 1.17 |
| 8 | III | 6.8 | 203 | 123 | 1.17 | 0.56 | 4.6 | 6.0 | 0.04 | 7.4 | 208 | 129 | 1.21 |
| 9 | | 5.6 | 152 | 109 | 0.80 | 0.56 | 4.6 | 6.0 | 0.04 | 6.2 | 157 | 115 | 0.84 |
| 10 | | 5.5 | 168 | 99 | 1.41 | 0.56 | 4.6 | 6.0 | 0.04 | 6.1 | 173 | 105 | 1.45 |
| 11 | | 5.1 | 171 | 100 | 1.12 | 0.55 | 2.4 | 5.8 | 0.30 | 5.7 | 173 | 106 | 1.42 |
| 12 | IV | 5.6 | 128 | 121 | 1.18 | 0.55 | 2.4 | 5.8 | 0.30 | 6.2 | 130 | 127 | 1.48 |
| 13 | | 4.5 | 123 | 120 | 1.12 | 0.55 | 2.4 | 5.8 | 0.30 | 5.1 | 125 | 126 | 1.42 |
| 14 | | 4.4 | 117 | 107 | 0.77 | 0.55 | 2.4 | 5.8 | 0.30 | 5.0 | 119 | 113 | 1.07 |
| 15 | | 6.7 | 161 | 76 | 3.58 | 0.92 | 0.89 | 7.7 | 5.9 | 7.6 | 162 | 84 | 9.5 |
| 16 | V | 9.2 | 177 | 68 | 5.07 | 0.92 | 0.89 | 7.7 | 5.9 | 10.1 | 178 | 76 | 11.0 |
| 17 | | 10.2 | 131 | 63 | 6.35 | 0.92 | 0.89 | 7.7 | 5.9 | 11.1 | 132 | 71 | 12.3 |
| 18 | | 9.8 | 103 | 63 | 6.70 | 0.92 | 0.89 | 7.7 | 5.9 | 10.7 | 104 | 71 | 12.6 |
| | | Intake | | | | Balance | | | | | | | |
| 1 | | 7.6 | 128 | 48 | 14 | −4.1 | +18 | −22 | − 5.3 | | | | |
| 2 | I | 11.9 | 144 | 77 | 30 | −0.4 | + 4 | + 1 | + 4.0 | | | | |
| 3 | | 9.1 | 275 | 62 | 21 | −2.4 | +76 | −28 | − 0.8 | | | | |
| 4 | | 4.3 | 106 | 32 | 9.3 | −7.6 | −111 | −50 | −13.9 | | | | |
| 5 | II | 5.7 | 148 | 115 | 0.2 | −5.3 | +17 | +38 | −15.7 | | | | |
| 6 | | 6.9 | 180 | 142 | 0.7 | −2.5 | +27 | +71 | −11.1 | | | | |
| 7 | | 6.1 | 181 | 139 | 0.6 | −0.4 | + 6 | + 1 | − 0.6 | | | | |
| 8 | III | 6.9 | 180 | 142 | 0.7 | −0.5 | −28 | +13 | − 0.5 | | | | |
| 9 | | 6.0 | 150 | 115 | 0.5 | −0.2 | − 7 | 0 | − 0.3 | | | | |
| 10 | | 5.8 | 152 | 117 | 0.5 | −0.3 | −21 | +12 | − 1.0 | | | | |
| 11 | | 5.7 | 150 | 116 | 0.4 | 0.0 | −23 | +10 | − 1.0 | | | | |
| 12 | IV | 5.7 | 149 | 117 | 0.7 | −0.5 | +19 | −10 | − 0.8 | | | | |
| 13 | | 5.8 | 149 | 117 | 1.0 | +0.7 | +24 | − 9 | − 0.4 | | | | |
| 14 | | 5.7 | 148 | 118 | 0.7 | +0.7 | +29 | + 5 | − 0.4 | | | | |
| 15 | | 9.2 | 156 | 70 | 17 | +1.6 | − 6 | −14 | + 7.5 | | | | |
| 16 | V | 11.6 | 141 | 55 | 18 | +1.5 | −37 | −21 | + 7.0 | | | | |
| 17 | | 11.3 | 151 | 61 | 32 | +0.2 | +19 | −10 | +19.7 | | | | |
| 18 | | 6.0 | 113 | 41 | 10 | −4.7 | + 9 | −30 | − 2.6 | | | | |

sis of combined two, four, or five day collections as defined by the length of the metabolic periods. Carmine red was not employed to limit the stool periods, and the interpretation of the results is apparent without this technique.

*Serum chemistries and electrocardiograms.* During the progress of these studies repeated analyses were carried out for serum magnesium, sodium, potassium, chloride, calcium, phosphorus, total protein, A/G ratio, pH and hematocrit. No influence of the artificial dietary routine was discernible in these determinations, and they will not be referred to again.

Repeated electrocardiograms in Cases 1 and 2 likewise were unchanged from their normal patterns by the dietary routine.

*Analytical techniques.* Stool analyses were carried out as described by Wallace, Holliday, Cushman, and Elkinton (33) whereby an aliquot of an aqueous homogenate is employed for nitrogen analysis (34), and an aliquot is treated with nitric acid for complete extraction of base. This total extraction of base by nitric acid has been validated by others (35), and the sodium and potassium were determined by flame spectrophotometry (36) and the magnesium by the titan yellow dye method (37). The precision of the magnesium assays is satisfactory, giving a coefficient of variation of 2 per cent calculated from 23 replicate stool analyses. The accuracy is less readily documented since the titan yellow dye method is sensitive to unusual concentrations of other ions commonly found in biological material (38,

39). Each general set of conditions must be tested, and we have invariably found less than a 10 per cent difference between known and determined values if certain unusual ionic ratios are excluded. The urine analyses for sodium and potassium were performed on diluted urine by flame spectrophotometry, and the magnesium analyses were completed on samples of urine that were dried, ashed in platinum in a muffle oven at 550° C., and extracted with hydrochloric acid inasmuch as certain substances in fresh urine were noted to have an inconstant effect on the coupling of the magnesium with the titan yellow dye. These analyses coincided with a change in some of the techniques usually employed in the laboratory so that replicate studies by different methods were carried out to confirm their reliability. The precision and accuracy of the chemical techniques as evaluated elsewhere (40) fall well within those of the balance method.

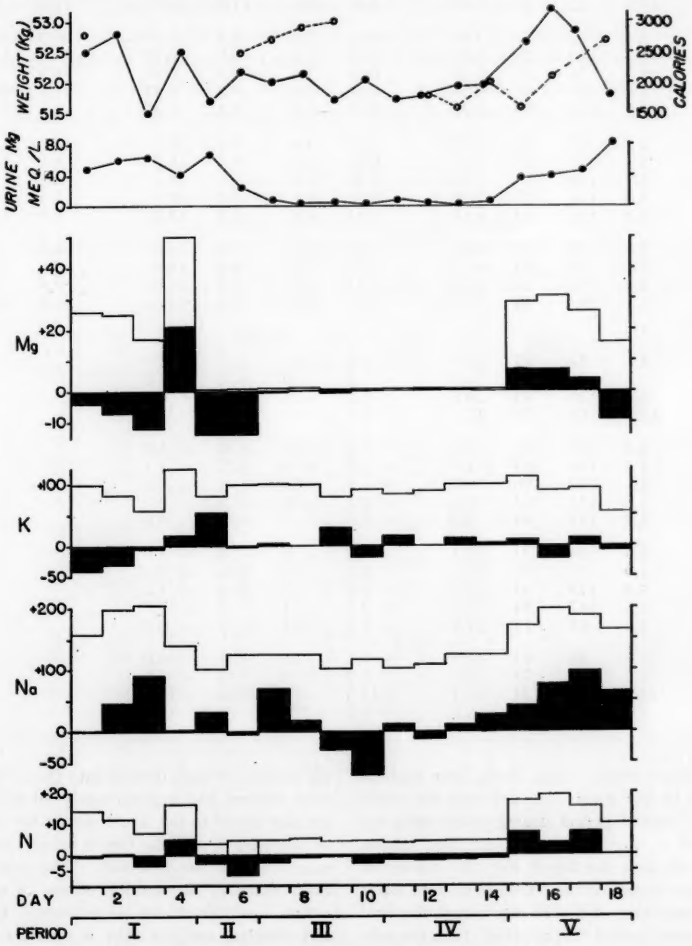



FIG. 1. METABOLIC BALANCE IN CASE 1

The caloric intake is represented by the connected series of solid points, and the available daily weights are represented by circles. In the areas representing the mineral and nitrogen balances the minerals are expressed in mEq. and the nitrogen in Gm. The intake is represented by the line running across the chart above the zero line, and the positive or negative balance is indicated by the solid black area. For the actual values and for the partition of the excretion between the urine and stool, the tables should be consulted.

TABLE III

*Daily nitrogen and mineral balances of Case 3*

*(N expressed in Gm.; Na, K, Mg in mEq.)*

| Day | Period | N | Na | K | Mg | N | Na | K | Mg | N | Na | K | Mg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Urine | | | | Stool | | | | Total output | | | |
| 1 | | 10.7 | 100 | 43 | 5.90 | 1.49 | 4.6 | 11.9 | 13.7 | 12.2 | 105 | 55 | 19.6 |
| 2 | I | 11.2 | 91 | 54 | 5.79 | 1.49 | 4.6 | 11.9 | 13.7 | 12.7 | 96 | 66 | 19.5 |
| 3 | | 11.2 | 80 | 54 | 6.51 | 1.49 | 4.6 | 11.9 | 13.7 | 12.7 | 85 | 66 | 20.2 |
| 4 | | 17.1 | 82 | 50 | 7.67 | 1.49 | 4.6 | 11.9 | 13.7 | 18.6 | 87 | 62 | 21.4 |
| 5 | | 8.4 | 62 | 63 | 3.93 | 0.61 | 2.8 | 5.8 | 0.47 | 9.0 | 65 | 69 | 4.40 |
| 6 | II | 6.7 | 130 | 79 | 1.77 | 0.61 | 2.8 | 5.8 | 0.47 | 7.3 | 133 | 85 | 2.24 |
| 7 | | 4.2 | 74 | 95 | 0.39 | 0.61 | 2.8 | 5.8 | 0.47 | 4.8 | 77 | 101 | 0.86 |
| 8 | | 4.8 | 101 | 92 | 0.36 | 0.61 | 2.8 | 5.8 | 0.47 | 5.4 | 104 | 98 | 0.83 |
| 9 | | 4.9 | 116 | 90 | 0.32 | 0.61 | 2.8 | 5.8 | 0.47 | 5.5 | 119 | 96 | 0.79 |
| 10 | | 4.4 | 123 | 86 | 0.38 | 0.83 | 4.4 | 9.1 | 18.1 | 5.2 | 127 | 95 | 18.5 |
| 11 | III | 4.3 | 140 | 56 | 2.12 | 0.83 | 4.4 | 9.1 | 18.1 | 5.1 | 144 | 65 | 20.2 |
| 12 | | 4.7 | 138 | 76 | 5.10 | 0.83 | 4.4 | 9.1 | 18.1 | 5.5 | 142 | 85 | 23.2 |
| 13 | | 4.6 | 114 | 87 | 6.10 | 0.83 | 4.4 | 9.1 | 18.1 | 5.4 | 118 | 96 | 24.2 |
| 14 | | 4.1 | 144 | 95 | 6.19 | 0.83 | 4.4 | 9.1 | 18.1 | 4.9 | 148 | 104 | 24.3 |
| 15 | | 5.6 | 85 | 45 | 8.30 | 1.23 | 0.93 | 9.4 | 16.6 | 6.8 | 86 | 54 | 24.9 |
| 16 | IV | 7.4 | 51 | 52 | 6.11 | 1.23 | 0.93 | 9.4 | 16.6 | 8.6 | 52 | 61 | 22.7 |
| 17 | | 8.6 | 78 | 46 | 4.38 | 1.23 | 0.93 | 9.4 | 16.6 | 9.8 | 79 | 55 | 21.0 |
| 18 | | 15.4 | 192 | 74 | 7.53 | 1.23 | 0.93 | 9.4 | 16.6 | 16.6 | 193 | 83 | 24.1 |
| | | Intake | | | | Balance | | | | | | | |
| 1 | | 11.6 | 54 | 47 | 13.7 | − 0.6 | −51 | − 8 | − 5.9 | | | | |
| 2 | I | 11.3 | 76 | 64 | 16.9 | − 1.4 | −20 | − 2 | − 2.6 | | | | |
| 3 | | 16.9 | 59 | 95 | 20.5 | + 4.2 | −26 | +29 | + 0.3 | | | | |
| 4 | | 13.7 | 64 | 59 | 17.1 | − 4.9 | −23 | − 3 | − 4.3 | | | | |
| 5 | | 5.4 | 136 | 103 | 0.4 | − 3.6 | +71 | +34 | − 4.0 | | | | |
| 6 | II | 5.5 | 136 | 107 | 1.1 | − 1.8 | + 3 | +22 | − 1.1 | | | | |
| 7 | | 5.1 | 133 | 106 | 0.8 | + 0.3 | +56 | + 5 | − 0.1 | | | | |
| 8 | | 5.5 | 134 | 103 | 0.8 | + 0.1 | +30 | + 5 | 0.0 | | | | |
| 9 | | 4.5 | 117 | 94 | 0.8 | − 1.0 | − 2 | − 2 | 0.0 | | | | |
| 10 | | 4.9 | 119 | 91 | 22.3 | − 0.3 | − 8 | − 4 | + 3.8 | | | | |
| 11 | III | 4.9 | 119 | 91 | 22.3 | − 0.2 | −25 | +26 | + 2.1 | | | | |
| 12 | | 4.9 | 119 | 91 | 22.0 | − 0.6 | −23 | + 6 | − 1.2 | | | | |
| 13 | | 4.5 | 117 | 94 | 22.3 | − 0.9 | − 1 | − 2 | − 1.9 | | | | |
| 14 | | 3.6 | 95 | 73 | 17.5 | − 1.3 | −53 | −31 | − 6.8 | | | | |
| 15 | | 16.3 | 58 | 93 | 18.9 | + 9.5 | −28 | +39 | − 6.0 | | | | |
| 16 | IV | 15.8 | 61 | 78 | 20.7 | + 7.2 | + 9 | +17 | − 2.0 | | | | |
| 17 | | 23.1 | 154 | 121 | 31.7 | +13.3 | +75 | +66 | +10.7 | | | | |
| 18 | | 7.4 | 71 | 33 | 8.9 | − 9.2 | −122 | −50 | −15.2 | | | | |

*Program of balance study.* Each of the four subjects was placed on an 18 day study. In each case the initial four days were a control period during which time the subjects consumed a simple diet of their own choice. During the next 10 days the liquid diet was consumed, together with a few inconsequential food items and water *ad lib.* At the conclusion of the 10 day period the final 4 days were a control period during which time the subjects again consumed a simple diet of their own choice. The middle 10 days in the first two cases were divided into three metabolic periods: an initial "transitional" 2 day period and two subsequent 4 day periods. The data from these two cases establish a response to a 10 day dietary intake virtually free of magnesium. To avoid confounding the effects of no magnesium intake with that of an artificial, liquid diet the last two subjects had the middle 10 days divided into two 5 day periods. The third subject had approximately 20 mEq. of magnesium per day added to the liquid diet in the second half of the 10 day period, and the fourth subject had this additional magnesium in the first half. The data from these two cases establish the specific effects of magnesium deprivation apart from the experimental routine. Subjects and chemical analysts alike were not aware of the magnesium content of the diet.

## RESULTS

The data of the four balance studies are presented in five tables and two charts. Tables I and II present the data from Cases 1 and 2, who were

on the low magnesium diet for 10 days. The low nitrogen content of the diet prevented the subjects maintaining the nitrogen intake at the levels of the first and last metabolic periods. While on the low magnesium diet Case 1 consumed 4.6 Gm. of nitrogen or 29 Gm. of protein a day (0.55 Gm. protein per Kg.). In Case 2 the nitrogen intake averaged 6.0 Gm. or 38 Gm. protein per day (0.50 Gm. protein per Kg.). These intakes are within the range of protein requirements in young adults as determined in the studies of Hegsted, Tsongas, Abbott, and Stare (41) and of Bricker, Mitchell, and Kinsman (42). The minor fluctuations in the magnesium intake are due to the insignificant magnesium content of additional foods permitted during the liquid diet. In both cases following the institution of the low magnesium routine a striking and prompt decrease in the magnesium content of the urine and stool took place. Within two to three days a maximum conservation of magnesium in the urine was achieved, and after the transitional Period II negligible amounts of magnesium appeared in the stool. In the first and fifth periods approximately one-third to one-half of the magnesium output appeared in the urine with the remainder in the stool. Aside from the

TABLE IV

*Daily nitrogen and mineral balances of Case 4*

*(N expressed in Gm.; Na, K, Mg in mEq.)*

| Day | Period | N | Na | K | Mg | N | Na | K | Mg | N | Na | K | Mg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Urine | | | | Stool | | | | Total output | | | |
| 1 | | 15.6 | 120 | 51 | 10.1 | 1.52 | 1.92 | 17.9 | 15.0 | 17.2 | 122 | 69 | 25.1 |
| 2 | I | 17.2 | 118 | 63 | 8.78 | 1.52 | 1.92 | 17.9 | 15.0 | 18.7 | 120 | 81 | 23.8 |
| 3 | | 16.3 | 146 | 56 | 7.87 | 1.52 | 1.92 | 17.9 | 15.0 | 18.7 | 148 | 74 | 22.8 |
| 4 | | 12.1 | 62 | 45 | 8.13 | 1.52 | 1.92 | 17.9 | 15.0 | 13.6 | 64 | 63 | 23.1 |
| 5 | | 8.8 | 116 | 39 | 5.98 | 0.37 | 3.04 | 7.3 | 5.1 | 9.2 | 119 | 46 | 11.1 |
| 6 | II | 6.0 | 98 | 43 | 2.96 | 0.37 | 3.04 | 7.3 | 5.1 | 6.3 | 101 | 50 | 8.1 |
| 7 | | 5.4 | 126 | 82 | 4.19 | 0.37 | 3.04 | 7.3 | 5.1 | 5.8 | 129 | 89 | 9.3 |
| 8 | | 4.0 | 80 | 63 | 3.74 | 0.37 | 3.04 | 7.3 | 5.1 | 4.4 | 83 | 70 | 8.8 |
| 9 | | 4.6 | 114 | 88 | 5.41 | 0.37 | 3.04 | 7.3 | 5.1 | 5.0 | 117 | 95 | 10.5 |
| 10 | | 4.9 | 110 | 84 | 3.42 | 0.69 | 2.43 | 14.7 | 4.2 | 5.6 | 113 | 98 | 7.6 |
| 11 | III | 4.8 | 136 | 67 | 2.14 | 0.69 | 2.43 | 14.7 | 4.2 | 5.5 | 139 | 82 | 6.3 |
| 12 | | 5.1 | 127 | 86 | 1.06 | 0.69 | 2.43 | 14.7 | 4.2 | 5.8 | 130 | 100 | 5.3 |
| 13 | | 5.6 | 169 | 78 | 1.39 | 0.69 | 2.43 | 14.7 | 4.2 | 6.3 | 172 | 92 | 5.6 |
| 14 | | 4.9 | 116 | 100 | 0.73 | 0.69 | 2.43 | 14.7 | 4.2 | 5.6 | 118 | 115 | 4.9 |
| 15 | | 5.8 | 135 | 59 | 2.05 | 0.96 | 0.72 | 12.2 | 5.6 | 6.8 | 135 | 71 | 7.7 |
| 16 | IV | 10.5 | 182 | 66 | 5.95 | 0.96 | 0.72 | 12.2 | 5.6 | 11.4 | 182 | 78 | 11.6 |
| 17 | | 9.1 | 133 | 37 | 6.05 | 0.96 | 0.72 | 12.2 | 5.6 | 10.1 | 133 | 49 | 11.7 |
| 18 | | 10.4 | 152 | 77 | 7.20 | 0.96 | 0.72 | 12.2 | 5.6 | 11.4 | 153 | 89 | 12.8 |
| | | Intake | | | | Balance | | | | | | | |
| 1 | | 18.6 | 85 | 65 | 20.2 | + 1.4 | −37 | − 4 | − 4.9 | | | | |
| 2 | I | 19.7 | 101 | 79 | 26.5 | + 1.0 | −19 | − 2 | + 2.7 | | | | |
| 3 | | 20.7 | 81 | 56 | 14.6 | + 2.0 | −67 | −18 | − 8.2 | | | | |
| 4 | | 12.7 | 54 | 59 | 21.1 | − 0.9 | −10 | − 4 | − 2.0 | | | | |
| 5 | | 5.1 | 133 | 103 | 24.5 | − 4.1 | +14 | +57 | +13.4 | | | | |
| 6 | II | 4.1 | 100 | 76 | 18.5 | − 2.2 | − 1 | +26 | +10.4 | | | | |
| 7 | | 5.7 | 138 | 104 | 25.0 | − 0.1 | + 9 | +15 | +15.7 | | | | |
| 8 | | 4.1 | 105 | 82 | 19.4 | − 0.3 | +22 | +12 | +10.6 | | | | |
| 9 | | 4.8 | 114 | 86 | 20.9 | − 0.2 | − 3 | − 9 | +10.4 | | | | |
| 10 | | 4.8 | 119 | 95 | 1.2 | − 0.8 | + 6 | − 3 | − 6.4 | | | | |
| 11 | III | 5.5 | 136 | 104 | 0.9 | 0.0 | − 3 | +22 | − 5.4 | | | | |
| 12 | | 5.7 | 135 | 105 | 0.9 | − 0.1 | + 5 | + 5 | − 4.4 | | | | |
| 13 | | 5.7 | 138 | 104 | 0.7 | − 0.6 | −34 | +12 | − 4.9 | | | | |
| 14 | | 4.8 | 120 | 92 | 0.5 | − 0.8 | + 2 | −23 | − 4.4 | | | | |
| 15 | | 14.7 | 90 | 83 | 19.2 | + 7.9 | −45 | +12 | +11.5 | | | | |
| 16 | IV | 23.2 | 142 | 96 | 28.4 | +11.8 | −40 | +18 | +16.8 | | | | |
| 17 | | 12.6 | 81 | 73 | 26.3 | + 2.5 | −52 | +24 | +14.6 | | | | |
| 18 | | 14.3 | 139 | 50 | 18.6 | + 2.9 | −14 | −39 | + 5.8 | | | | |

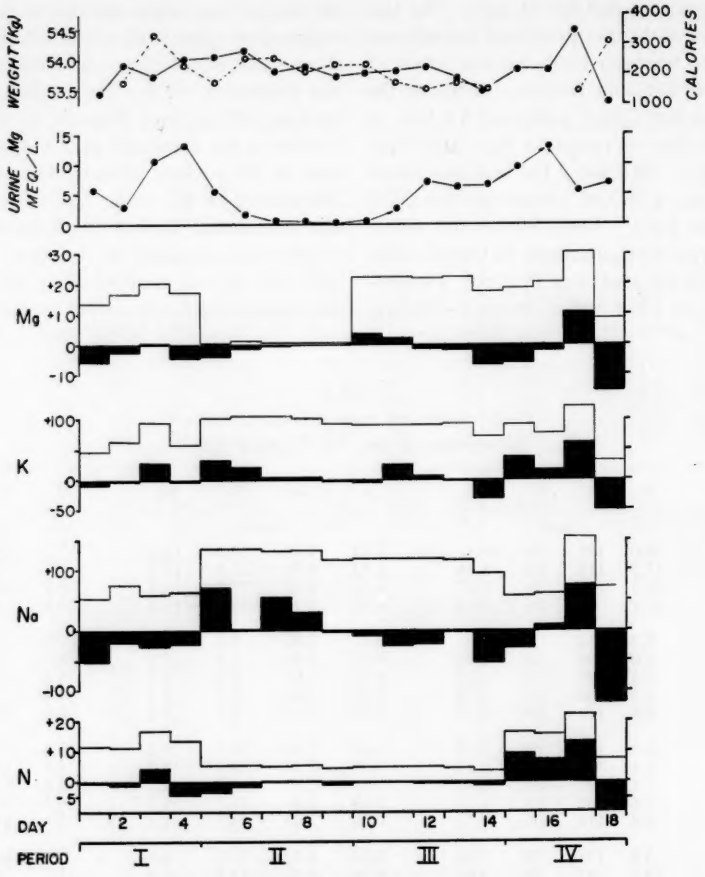



FIG. 2. METABOLIC BALANCE IN CASE 3
Charting convention as in Figure 1.

magnesium changes the balance data do not show any consistent or revealing trends in the other components except for the slightly negative nitrogen balance. This loss is presumably a consequence of the generous nitrogen intake maintained by these subjects normally leading to a release of this element when on a low intake. The urinary conservation of magnesium was less effective in Case 2, but these two studies are in good agreement. The data of Case 1 are charted in Figure 1 where the intake in relation to the balance may be seen. The charting convention employed is similar to that of Moore and Ball (43) and is explained in the legend. The partition of the excretion between the urine and the stool has been omitted for the sake of clarity.

The data of Cases 3 and 4 are presented in Tables III and IV, and they establish the specific effect of a low magnesium intake apart from that of the artificial, liquid diet. A prompt urinary conservation of magnesium appeared more clearly in Case 3 than in Case 4, and it was associated with a marked decline in the stool excretion. The magnesium content of the stools in Case 4 in Period III is a consequence of the carry-over into this period of the residual feces formed while on the high magnesium intake. Aside from the magnesium changes the balance data do not reveal

any consistent trends except for the negative nitrogen balance also seen in the first two cases. Case 3 had a negative magnesium balance throughout the entire study in contrast to Case 4 where a positive balance developed in Periods II and IV when an adequate intake was present. The lag in the stool excretion of magnesium does not appear to account for the positive balances of metabolic Period II. In Figure 2 the data in Case 3 are displayed using the same conventions as explained in the legend of Figure 1.

While on the processed diet the daily calcium intake amounted to 0.3 to 0.5 mM in these four subjects, and prior to this study the dietary habits of the volunteers included the generous calcium intake seen in most urban American diets.

Table V records the serial weights and caloric intakes of the four subjects.

## DISCUSSION

This study was directed towards the determination of the magnitude of the obligatory loss of magnesium in normal individuals while on a diet sufficient in other respects. Expect for the study of Fitzgerald and Fourman (21) we are not aware of previous work determining the obligatory magnesium excretion in humans. These authors have reported the application to two humans of an artificial diet with the daily content of 1.1 mEq. of magnesium over a period of three weeks. The effects of this diet were compounded in one subject by the use of a polystyrene sulphonate resin (25 per cent potassium salt and 75 per cent ammonium salt), thereby producing an augmented loss of divalent cations from the gastrointestinal tract. The urinary losses and stool losses of magnesium, apart from those caused by the resin binding of this element, were 1 to 2 mEq. per day, and the total depletion in approximately three weeks was found to be 46 mEq. of magnesium in one subject and 72 mEq. of magnesium in the subject who took the resin. There were no unusual symptoms or signs noted in these individuals at the conclusion of the study.

A feature of the present study is the low nitrogen and calcium intake while on the magnesium-free routine. Therefore, more correctly the results are concerned with the ability of healthy, young, female adults to conserve magnesium while on a normal caloric intake, low nitrogen intake, and a low calcium intake. It is possible that the intakes of nitrogen and calcium may alter the response of the individual to magnesium deprivation and that the conservation of magnesium would be different with other diets. Previous work in animals (44) demonstrates a possible relation between calcium and magnesium in this regard, and our experience is not extensive enough to test this hypothesis in humans. With these restrictions it is clear that the kidney by means of unknown mechanisms is very sensitive to the magnesium intake and promptly suppresses the urinary

TABLE V

*Weight and caloric intake of subjects*

| Day | Case 1 | | Case 2 | | Case 3 | | Case 4 | |
|---|---|---|---|---|---|---|---|---|
| | Kg. | Calories | Kg. | Calories | Kg. | Calories | Kg. | Calories |
| 1 | 52.8 | 2,590 | 76.8 | 1,580 | | 1,400 | 56.8 | 2,510 |
| 2 | | 2,850 | | 3,230 | 53.6 | 2,390 | 56.5 | 2,650 |
| 3 | | 1,520 | | 1,780 | 54.4 | 1,980 | 56.1 | 1,870 |
| 4 | | 2,610 | | 1,000 | 53.9 | 2,520 | 56.4 | 2,440 |
| 5 | | 1,720 | 76.0 | 2,230 | 53.6 | 2,600 | 56.5 | 2,190 |
| 6 | 52.5 | 2,230 | 75.9 | 2,770 | 54.0 | 2,780 | 56.1 | 1,800 |
| 7 | 52.7 | 2,080 | 76.4 | 2,870 | 54.0 | 2,100 | 56.4 | 3,020 |
| 8 | 52.9 | 2,150 | 75.9 | 2,770 | 53.8 | 2,210 | 55.0 | 1,590 |
| 9 | 53.0 | 1,730 | | 2,680 | 53.9 | 1,950 | 55.9 | 2,330 |
| 10 | | 2,050 | | 2,580 | 53.9 | 2,050 | 56.1 | 2,090 |
| 11 | | 1,720 | | 2,430 | 53.6 | 2,100 | 56.1 | 2,330 |
| 12 | 51.8 | 1,790 | 75.3 | 2,460 | 53.5 | 2,250 | 56.5 | 2,650 |
| 13 | 51.6 | 1,950 | 75.1 | 2,340 | 53.6 | 1,860 | 55.7 | 2,940 |
| 14 | 52.0 | 1,950 | | 2,300 | 53.5 | 1,430 | 55.7 | 2,250 |
| 15 | 51.6 | 2,650 | | 1,320 | | 2,160 | 56.4 | 2,950 |
| 16 | 52.1 | 3,170 | | 1,660 | | 2,150 | 56.0 | 3,600 |
| 17 | | 2,830 | | 2,840 | 53.5 | 3,450 | 56.1 | 2,380 |
| 18 | 52.7 | 1,800 | 74.4 | 1,060 | 54.3 | 1,120 | 55.9 | 2,530 |

loss when the intake drops to minimal levels. The analytical techniques for serum magnesium are not sufficiently precise today to define slight fluctuations in the serum magnesium that may play a role in the initiation of the conservation. As mentioned previously all the serum chemistries checked during this study were not significantly altered. Furthermore, there is no significant obligatory loss of magnesium in the stool. It is of interest to recall that the normal volume of gastrointestinal juices of 7 to 9 liters a day would deliver to the lumen of the bowel approximately 14 to 18 mEq. of magnesium. This is notably greater than the daily stool loss of magnesium seen in Cases 1 and 2. These facts together with the previous study of Fitzgerald and Fourman (21) indicate why a primary magnesium deficiency is not a common syndrome in humans provided the conserving mechanisms are intact.

The retention of magnesium may be compared to that of other cations more extensively studied. In experiments on the calcium excretion of normal individuals on low calcium diets, Bauer, Albright, and Aub (1) noted a loss of 10 mEq. of calcium per day in the feces and 3.2 mEq. per day in the urine. This study was generally accepted as defining an obligatory loss and carried the implication that conservation would be no better on calcium-free diets. Contrariwise, the work of Steggerda and Mitchell (5) and that of Hegsted, Moscoso, and Collazos (6) imply that the previous dietary intake of calcium is an important factor in the response of the body to low calcium intakes and that an apparent obligatory loss of calcium exists only if an individual has been conditioned by high intakes. To our knowledge calcium balances in human beings, employing a dietary routine with a calcium intake of less than 2 mEq. per day, have not been studied. The calcium balances of these and other subjects are a part of more extended observations being made at the present time.

The conservation of sodium in the urine during a low sodium diet has been studied by Leaf and Couter (8). They alternated an intake of a low sodium diet containing 9 mEq. per day with a high sodium diet containing approximately 450 mEq. per day. The low sodium diet did not initiate a prompt decrease in the renal loss of sodium, but within five or six days the renal excretion had diminished to 1 to 2 mEq. per 24 hours. The subjects lost weight at first, and the data demonstrate that the maximum renal conservation of sodium was not achieved until the reserves of extracellular fluid had been moderately depleted. This lag in renal conservation is presumably due to a delayed response of the adrenal glands or its activating mechanisms to sodium restriction.

The conservation of potassium is in some measure tested by balance studies of individuals on low potassium diets (9, 10, 13–15), but these studies did not achieve an insignificant potassium intake or a specific potassium deficiency in the diet so that the results do not afford the best evidence of the ability of the body to conserve this mineral. Evans, Hughes Jones, Milne, and Steiner (16) reported a study of electrolyte excretion during experimental potassium depletion obtained by feeding a subject a cation exchange resin. The daily fecal loss was approximately 25 mEq. due to the binding of potassium, and a net loss from the gastrointestinal tract ensued since the daily oral intake was only 7 mEq. An additional loss of potassium occurred in the urine where over 20 mEq. was being excreted daily in spite of the sizable negative balance. Fourman (17) gave a subject large amounts of a sulfonic acid exchange resin in the ammonium cycle, but this produced such a pronounced acidosis that only rather restricted conclusions may be drawn concerning the conservation of potassium on such a depleting routine. Black and Milne (12) placed two subjects on a low potassium intake by treating milk with a cation exchange resin to reduce the potassium content to 3 mEq. per liter and, incidentally, the magnesium content to 5 mEq. per liter. Over the seven days of observation the daily urinary potassium fell from approximately 50 mEq. to between 10 and 15 mEq. Other components of the diet were present in satisfactory amounts, and this study is a convincing example of a pure potassium depletion. The fact was clearly established that renal potassium conservation was not effective in preventing a potassium deficit developing quite rapidly. Squires and Huth (19), in an abbreviated report of a low potassium diet study, have demonstrated more effective renal conservation of this element with only 5 mEq. per day appearing in the urine on a daily intake of less than 1 mEq. Certain of these studies and other data on

potassium deficiency have been reviewed (18), and the inadequacies of renal potassium conservation are generally recognized.

The magnesium balance studies reported in this paper provide suggestive, but not conclusive, evidence in favor of a considerable revision downward of the magnesium requirements generally postulated for adult maintenance (4, 23, 26–28, 30). If the conserving mechanisms noted to be effective in these studies over a 10 day period were assumed to be permanent, an intake of less than 1 mEq. of magnesium a day would be sufficient for maintenance. However, the short duration of the study and the completeness of the diet in other respects make such speculations concerning the daily requirement of theoretical more than practical significance. The extent of our efforts and of others to achieve a magnesium-free intake demonstrates the slight chance of such a deficiency developing assuming relatively normal gastrointestinal function and a diet that is sufficient in calories and nitrogen.

Any definitive statement of the magnesium requirements should also consider such losses as arise from the skin and its appendages. These have not been determined in this study. If balance techniques were sufficiently precise, the magnesium requirements would be better defined by a method introduced by Leitch (45) in the study of calcium requirements. This approach would determine the regression line of magnesium intake on balance at different intake levels, including "negative" values obtained by the use of resin *per os*. Until such extended investigations are completed, we suspect the daily magnesium requirement of the adult is considerably less than appreciated formerly, and it may be in the vicinity of 1 mEq. The requirements of growth, convalescence, and pregnancy are, of course, a different problem.

## SUMMARY

The application of a liquid diet containing less than 0.12 mEq. of magnesium per liter (approximately 1 part per million by weight) to four volunteers permitted a study of the conservation of magnesium by humans over a 10 day period. Renal conservation prevented maximum losses in excess of 1.5 mEq. per day, and on the average less than 1.0 mEq. of magnesium per day was excreted in the urine. Stool excretion of magnesium while on the liquid diet was negligible after a transitional period.

Magnesium, like sodium, is a component of the body than can be rather rigidly conserved in the presence of a specific deficiency. This is in contrast to potassium where an obligatory loss of significance has been established in essentially normal circumstances.

The adult maintenance requirement may approximate 1 mEq. of magnesium per day.

## ACKNOWLEDGMENTS

The authors are particularly indebted to E. Gordon, H. Filipak, J. Hardy, and G. Merrill for their detailed care and attention in the completion of these balance studies.

## REFERENCES

1. Bauer, W., Albright, F., and Aub, J. C. Studies of calcium and phosphorus metabolism. II. The calcium excretion of normal individuals on a low calcium diet, also data on a case of pregnancy. J. clin. Invest. 1929, **7**, 75.
2. Mitchell, H. H., and Curzon, E. G. The dietary requirement of calcium and its significance *in* Actualitiés Scientifiques et Industrielles, No. 771. Paris, Hermann and Co., 1939.
3. Steggerda, F. R., and Mitchell, H. H. Further experiments on the calcium requirement of adult man and the utilization of the calcium in milk. J. Nutr. 1941, **21**, 577.
4. McCance, R. A., and Widdowson, E. M. Mineral metabolism of healthy adults on white and brown bread dietaries. J. Physiol. 1942, **101**, **44**.
5. Steggerda, F. R., and Mitchell, H. H. Variability in the calcium metabolism and calcium requirements of adult human subjects. J. Nutr. 1946, **31**, 407.
6. Hegsted, D. M., Moscoso, I., and Collazos, C. A study of the minimum calcium requirements of adult men. J. Nutr. 1952, **46**, 181.
7. McCance, R. A. Experimental sodium chloride deficiency in man. Proc. roy. Soc. B. 1936, **119**, 245.
8. Leaf, A., and Couter, W. T. Evidence that renal sodium excretion by normal human subjects is regulated by adrenal cortical activity. J. clin. Invest. 1949, **28**, 1067.
9. Pearson, O. H., and Eliel, L. P. Experimental studies with ACTH and cortisone in patients with neoplastic disease *in* Recent Progr. Hormone Res. New York, Academic Press Inc., 1951, vol. 6, p. 373.
10. Reimer, A., Schoch, H. K., and Newburgh, L. H. Certain aspects of potassium metabolism. J. Amer. diet. Ass. 1951, **27**, 1042.
11. Fourman, P., and Ainley-Walker, K. M. S. Experimental depletion of potassium in man. Lancet 1952, **2**, 368.

12. Black, D. A. K., and Milne, M. D. Experimental potassium depletion in man. Clin. Sci. 1952, **11**, 397.
13. Lowe, K. G. Metabolic studies with protein-free, electrolyte-free diet in man. Clin. Sci. 1953, **12**, 57.
14. Blahd, W. H., and Bassett, S. H. Potassium deficiency in man. Metabolism 1953, **2**, 218.
15. Quinn, M., Kleeman, C. R., Bass, D. E., and Henschel, A. Nitrogen, water, and electrolyte metabolism on protein and protein-free low-calorie diets in man. II. Adequate water intake. Metabolism 1954, **3**, 68.
16. Evans, B. M., Hughes Jones, N. C., Milne, M. D., and Steiner, S. Electrolyte excretion during experimental potassium depletion in man. Clin. Sci. 1954, **13**, 305.
17. Fourman, P. Depletion of potassium induced in man with an exchange resin. Clin. Sci. 1954, **13**, 93.
18. Moore, F. D., Boling, E. A., Ditmore, H. B., Jr., Sicular, A., Teterick, J. E., Ellison, A. E., Hoye, S. J., and Ball, M. R. Body sodium and potassium. V. The relationship of alkalosis, potassium deficiency and surgical stress to acute hypokalemia in man. Metabolism 1955, **4**, 379.
19. Squires, R. D., and Huth, E. J. The effects of a very low potassium diet on the urine and blood of normal human subjects. Clin. Res. Proc. 1955, **3**, 127.
20. Osborne, T. B., and Mendel, L. B. The inorganic elements in nutrition. J. biol. Chem. 1918, **34**, 131.
21. Fitzgerald, M. G., and Fourman, P. An experimental study of magnesium deficiency in man. Clin. Sci. 1956, **15**, 635.
22. Tibbetts, D. M., and Aub, J. C. Magnesium metabolism in health and disease. II. The effect of the parathyroid hormone. J. clin. Invest. 1937, **16**, 503.
23. Clark, G. W. Studies in the mineral metabolisms of adult man. Univ. Calif. Publ. Physiol. 1926, **5**, 195.
24. Carswell, H. E., and Winter, J. E. The effects of high and prolonged magnesium lactate intake upon the metabolism of magnesium and calcium in man. J. biol. Chem. 1931, **93**, 411.
25. Fabrykant, M., and Lavollay, J. Technique pour l'étude des bilans d'entrée et sortie du phosphore, du calcium et du magnesium chez l'homme. Quelques résultats expérimentaux. Bull. sci. Pharm. 1932, **39**, 34.
26. Leichsenring, J. M., Norris, L. M., and Lamison, S. A. Magnesium metabolism in college women: Observations on the effect of calcium and phosphorus intake levels. J. Nutr. 1951, **45**, 477.
27. Greenberg, D. M. Mineral metabolism. Calcium, magnesium and phosphorus. Ann. Rev. Biochem. 1939, **8**, 269.
28. Stearns, G. Human requirements of calcium, phosphorus and magnesium. J. Amer. med. Ass. 1950, **142**, 478.
29. Barnes, B. A., Eddison, G. G., and Cope, O. Preparation and bioassay of a low magnesium liquid diet for human consumption. Surgery 1957, **42**, 643.
30. Sherman, H. C. Chemistry of Food and Nutrition, 8th ed. New York, The Macmillan Co., 1952.
31. Bowes, A. deP., and Church, C. F. Food Values of Portions Commonly Used, 7th ed. Philadelphia, College Offset Press, 1951.
32. McCance, R. A., and Widdowson, E. M. The Chemical Composition of Foods, 2nd ed. Brooklyn, Chemical Publishing Co., Inc., 1947.
33. Wallace, W. M., Holliday, M., Cushman, M., and Elkinton, J. R. Application of internal standard flame photometer to analysis of biologic material. J. Lab. clin. Med. 1951, **37**, 621.
34. Folin, O. Determination of non-protein nitrogen *in* Laboratory Manual of Biological Chemistry, 5th ed. New York, Appleton-Century, 1934, p. 265.
35. Lowry, O. H., and Hastings, A. B. Histochemical changes associated with aging. I. Methods and calculations. J. biol. Chem. 1942, **143**, 257.
36. White, J. U. Precision of a simple flame photometer. Analyt. Chem. 1952, **24**, 394.
37. Garner, R. J. Colorimetric determination of magnesium in plasma or serum by means of titan yellow. Biochem. J. 1946, **40**, 828.
38. Gillam, W. S. A photometric method for the determination of magnesium. Industr. engin. Chem. (Analyt.) 1941, **13**, 499.
39. Kunkel, H. O., Pearson, P. B., and Schweigert, B. S. The photoelectric determination of magnesium in body fluids. J. Lab. clin. Med. 1947, **32**, 1027.
40. Gordon, E. B. A comparison of certain standard techniques employed in the mineral analysis of biologic material. Unpublished data.
41. Hegsted, D. M., Tsongas, A. G., Abbott, D. B., and Stare, F. J. Protein requirements of adults. J. Lab. clin. Med. 1946, **31**, 261.
42. Bricker, M., Mitchell, H. H., and Kinsman, G. M. The protein requirements of adult human subjects in terms of the protein contained in individual foods and food combinations. J. Nutr. 1945, **30**, 269.
43. Moore, F. D., and Ball, M. R. The Metabolic Response to Surgery. Springfield, Charles C Thomas, 1952, p. 5.
44. Kruse, H. D., Schmidt, M. M., and McCollum, E. V. Studies on magnesium deficiency in animals. V. Changes in the mineral metabolism of animals following magnesium deprivation. J. biol. Chem. 1934, **106**, 553.
45. Leitch, I. The determniation of the calcium requirements of man. Nutr. Abstr. Rev. 1937, **6**, 553.

# STUDIES WITH RADIOACTIVE ENDOTOXIN. III. THE EFFECT OF TOLERANCE ON THE DISTRIBUTION OF RADIOACTIVITY AFTER INTRAVENOUS INJECTION OF *ESCHERICHIA COLI* ENDOTOXIN LABELED WITH $Cr^{51}$ [1]

By F. J. CAREY, A. I. BRAUDE,[2] AND M. ZALESKY

*(From the Department of Internal Medicine, Southwestern Medical School, Dallas, Texas)*

(Submitted for publication June 3, 1957; accepted November 21, 1957)

The repeated injection at frequent intervals of the same dose of a bacterial endotoxin is followed by gradual diminution in the severity of its toxic effects. The state of resistance that develops during the course of such injections has been designated *tolerance* and is considered to be independent of specific antibody formation, because tolerance for one endotoxin is effective against the toxic action of immunologically distinct endotoxins of other gram-negative bacilli (1–3). The importance of the reticuloendothelial system in the development of tolerance has been explored by Beeson (4, 5), who demonstrated that reticuloendothelial "blockade" not only abolished immunity to the Shwartzman reaction, but also tolerance to the febrile response of sublethal doses of bacterial pyrogens.

The heavy localization of radioactivity in the liver of animals injected intravenously with endotoxin labeled with $Cr^{51}$ has provided evidence that the reticuloendothelial system removes the endotoxin from their circulation (6). In rabbits receiving massive lethal doses of endotoxin intravenously, it was found that the localization of endotoxin in the liver was not enough to prevent the persistent circulation of endotoxin in high concentrations in the plasma for several hours. Thus, 30 to 40 per cent of the total amount of injected radioactivity was present in the circulation for nearly three hours after the injection of 6.0 mg. of radioactive endotoxin in rabbits. Because it seemed likely that these large amounts of circulating endotoxin were responsible for the damaging effects of endotoxin, the following study was undertaken to determine whether tolerant animals owed their resistance to an increased removal of lethal doses of endotoxin from the circulation by the liver and other organs rich in reticuloendothelial cells.

## METHODS

Tolerance was measured in rabbits by their resistance to the pyrogenic action of endotoxin and in mice by their resistance to its lethal action. In both species an attempt was made to assess the part played by humoral immunity (circulating antibody) in the development of tolerance and on the distribution of endotoxin. For this reason an effort was made to establish: 1) A tolerant state in which circulating precipitins to endotoxin were absent or low; and 2) a high titer of humoral antibodies in animals who lacked resistance to endotoxin. To accomplish the first objective (tolerance without circulating antibody) animals were given X-irradiation before initiating the injections of endotoxin required for tolerance; to accomplish the second (circulating antibody without tolerance) the injections of endotoxin were discontinued with the expectation that tolerance would lapse while antibody titer persisted at an elevated level. With these purposes in mind, we carried out distribution studies with massive lethal doses of radioactive endotoxin in the following groups of rabbits and mice: 1) normal, 2) tolerant, 3) irradiated tolerant, and 4) lapsed tolerant.

Distribution studies with small doses of endotoxin were also carried out in normal and tolerant animals (Groups 1 and 2) in order to learn whether or not the differences in distribution observed with lethal doses in these two groups would also be found with sublethal doses.

## EXPERIMENTAL

### *Massive lethal doses of endotoxin*

Albino rabbits weighing 2 to 3.5 kilograms and white Swiss mice weighing approximately 20 grams were obtained from dealers in Dallas and Austin, Texas. They were housed in air-conditioned quarters.

1. *Preparation of endotoxin.* The endotoxin was prepared from *Escherichia coli* grown in synthetic media and

[1] This work was supported by the Research and Development Division, Office of the Surgeon General, Department of the Army, under contract Number DA-49-007-MD-519.

[2] Reprint requests should be sent to A. I. Braude, Department of Medicine, University of Pittsburgh Medical School, Pittsburgh, Penna.

TABLE I

*Fever indices before and after a course of endotoxin*

| Rabbit no. | Start | Completion |
|---|---|---|
| 1 | 147 | 77 |
| 2 | 72 | 27 |
| 3 | 97 | 44 |
| 4 | 97 | 30 |
| 5 | 141 | 111 |
| 6 | 113 | 37 |
| 7 | 61 | 30 |
| 8 | 40 | 29 |
| 9 | 92 | 55 |
| 10 | 88 | 62 |
| 11 | 97 | 75 |
| 12 | 84 | 29 |
| 13 | 73 | 25 |
| 14 | 77 | 72 |
| 15 | 55 | 40 |
| 16 | 51 | 13 |
| 17 | 60 | 27 |
| 18 | 57 | 44 |
| 19 | 30 | 21 |
| 20 | 43 | 14 |
| 21 | 65 | 48 |
| Mean index: | 78 | 43.3 |

S. E. difference between means, 8.5
t, 4.1
$p < 0.01$

labeled by direct incubation with radioactive chromium (7). The procedure consisted of suspending the endotoxin in a phosphate buffer to give a concentration of 5.0 mg. per ml. and incubating at 37° C. with $Na_2Cr^{51}O_4$ for 48 hours. Then the suspension of endotoxin was dialyzed against distilled water at 4 to 8° C. until only insignificant amounts of radioactivity appeared in the dialysis bath. The toxicity ($LD_{50}$ in mice) was not changed upon labeling with $Cr^{51}$ in these experiments or in those reported earlier (7). The radioactivity of the labeled endotoxin at the time of injection ranged from 200 to 400 counts per second per mg.

2. *Injection of radioactive endotoxin.* Rabbits were inoculated in the ear vein with 6.0 mg.[3] $Cr^{51}$ labeled endotoxin suspended in 1.0 ml. of phosphate buffer at the time of the distribution studies. In mice the distribution of two different lethal doses of endotoxin was determined. One group of mice received 0.5 mg. of $Cr^{51}$ labeled endotoxin intracardially and a second group received 1.5 mg. by inoculation into the tail vein. Both doses were given in a volume of 0.25 ml. of phosphate buffer.

3. *Technique for producing tolerance.* In rabbits tolerance was readily established by the repeated injection of the same dose of endotoxin and manifested by a reduction in the amount of fever, weakness and listlessness elicited by that dose.

The schedule for producing tolerance in most rabbits was 11 or 12 injections during 27 days so that one injection was given every second or third day. The first three were given intraperitoneally and the remainder intravenously. The first two doses were 0.025 mg. and the remainder 0.05 mg.

Temperatures were measured rectally with a thermistor apparatus (E. H. Sargent and Co.). The instantaneous temperature records obtained by this device are more accurate than those which can be measured with the ordinary mercury bulb clinical thermometer. The fever index was calculated by measuring the area under the fever curve plotted over a six hour period. The index is expressed as the number of unit squares contained within a given area on graph paper divided by lines placed 2.5 mm. apart. The fever indices at the start and completion of the schedule of intravenous injections described above for 21 rabbits are shown in Table I.

These values represent a lower and less prolonged febrile response to the intravenous injection of endotoxin in tolerant rabbits. The well known alteration in the shape of the fever curve was also evident after tolerance was established (4). Thus, before tolerance the fever curve had two peaks; after tolerance, the second peak was diminished or failed to appear.

In 10 rabbits (Numbers 1 through 10), injections were discontinued for a period of five weeks. During this time, the titer of precipitins had fallen from 1 : 16 (average) at the time of the last injection to 1 : 4. Although these animals are referred to as "lapsed-tolerant," they tolerated the lethal injection of endotoxin better than normals as judged by their alertness, their ability to remain standing, their muscle tone when held, and the resistance offered upon removal from their cages. The fever index could not be applied as a measure of tolerance in these animals because another sublethal dose just before the tracer studies would nullify the effect of discontinuing injections.

A group of 12 rabbits was irradiated with 500 r (51 r per minute for 9.8 minutes) two days before beginning

TABLE II

*The effect of irradiation on the development of tolerance to injected endotoxin*

| Rabbit | Start | Completion |
|---|---|---|
| L | 126 | 86 |
| U | 128 | 116 |
| G | 134 | 98 |
| M | 82 | 82 |
| W | 104 | 32 |
| P | 114 | 64 |
| V | 120 | 96 |
| O | 126 | 96 |
| Z | 58 | 48 |
| X | 132 | 82 |
| S | 94 | 72 |
| R | 96 | 64 |
| Mean | 109.5 | 78 |

S. E. difference between means, 6.7
t, 4.8
$p < 0.01$

[3] Six mg. of endotoxin is equal to approximately 10 $LD_{50}$'s.

TABLE III

*The effect of tolerance on the $LD_{50}$ for endotoxin*

| Dose (mg.) | Actual mortality rate at 72 hrs. after challenge | | | |
|---|---|---|---|---|
| | Tolerant | | Normal | |
| | I.P.* | I.V.† | I.P.* | I.V.† |
| 4.0 | 5/5‡ | | | |
| 2.0 | 5/5 | 4/4 | | |
| 1.0 | 2/5 | 5/5 | 5/5 | |
| 0.5 | 1/5 | 9/10 | 5/5 | 5/5 |
| 0.25 | 0/5 | 4/10 | 4/5 | 8/9 |
| 0.125 | | 3/10 | 4/5 | 8/9 |
| 0.0625 | | 0/10 | 1/5 | 4/9 |
| 0.0312 | | 0/10 | 1/5 | 5/9 |
| 0.0156 | | | | 5/9 |
| 0.0078 | | | | 1/4 |
| 0.0039 | | | | 1/4 |

| Calculated $LD_{50}$'s | |
|---|---|
| Tolerant mice challenged intravenously | 0.25 mg. |
| Tolerant mice challenged intraperitoneally | 1.00 mg. |
| Normal controls challenged intravenously | 0.025 mg. |
| Normal controls challenged intraperitoneally | 0.097 mg. |

* I.P., intraperitoneal challenge.
† I.V., intravenous challenge.
‡ The numerator represents deaths and the denominator total number of mice inoculated with a given dose.

the series of injections leading to tolerance. The irradiation was given with 250 KV: 15 ma ½ Cu 1 Al filter at a distance of 78 cm. to the center of the carrier in which the rabbits were contained. At four and nine-tenths minutes, the positions of the two rabbits in each container were reversed. The fever indices at the start and completion of intravenous injections of endotoxin are shown in Table II.

4. *Technique for producing tolerance in mice.* One sublethal dose of endotoxin was injected intraperitoneally every other day for a total of six doses. The schedule consisted of 0.005 mg. of endotoxin for the first dose, 0.01 mg. for the second, and 0.02 mg. of endotoxin for the remaining four doses. This technique always produced tolerance which could be demonstrated by the high doses of endotoxin tolerated upon intravenous as well as intraperitoneal challenge. The results shown in Table III, for example, were obtained with this schedule of injections when the mice were challenged three days after the last injection. The mice weighed 20 grams and the intravenous injections were made in the tail vein. The $LD_{50}$'s, calculated by the method of Reed and Muench (8), after 72 hours are also shown.

These results disclose that this schedule increased the tolerance about 10 times over normal whether tolerance was measured intravenously or intraperitoneally. On the day of all tracer experiments with radioactive endotoxin the $LD_{50}$ was calculated in animals which had received simultaneously the same schedule of injections as the test animals.

Animals with lapsed tolerance were those in which the $LD_{50}$ had been permitted to fall approximately to normal through lapse of time after the last injections of endotoxin. The period required for tolerance to lapse was variable. In some animals tolerance persisted for two to three weeks with little change. In others, tolerance would disappear in this time. Enough extra mice were therefore incorporated into each group to permit periodic determinations of the degree of tolerance.

5. *Sacrifice of animals and preparation of tissues.* All rabbits and mice were sacrificed at the specified time intervals by exsanguination through the heart. Saline was given intravenously to rabbits during the exsanguination procedure so that the animals remained alive long enough to permit removal of maximum amounts of blood.

The tissues of all sacrificed animals were weighed and then soaked in tap water at 4° C. until the washings contained little or no hemoglobin in a 24 hour period. This procedure was necessary to insure that the radioactivity in an organ was not that of the blood contained within it. Admittedly, endotoxin could be lost from the tissues but it is unlikely that a large proportion would disappear in view of the finding that the total radioactivity in the combined washings did not exceed by more than 5 per cent of the total injected that amount calculated to be present in the blood which had been washed out. The amount of radioactivity in the blood washed out of the tissues was approximately equal to the difference between total circulating radioactivity and radioactivity in the sample removed by cardiac puncture.

The tissues of rabbits were then dried in an oven at 100° C. and digested with concentrated nitric acid. The tissues of mice were not digested, with the exception of

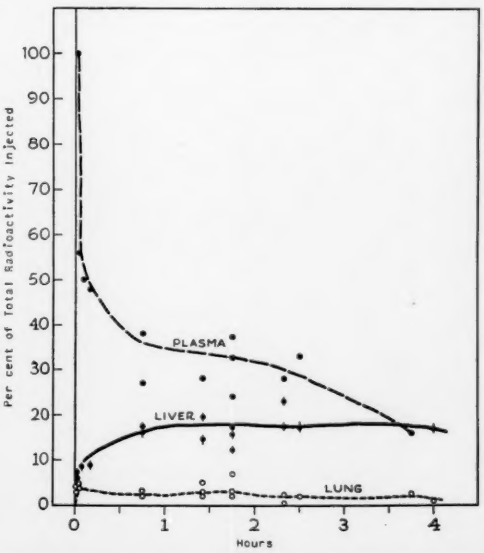



FIG. 1. DISTRIBUTION OF RADIOACTIVITY IN NORMAL RABBITS

Each normal rabbit received an injection of a massive lethal dose (6.0 mg.) of radioactive endotoxin.

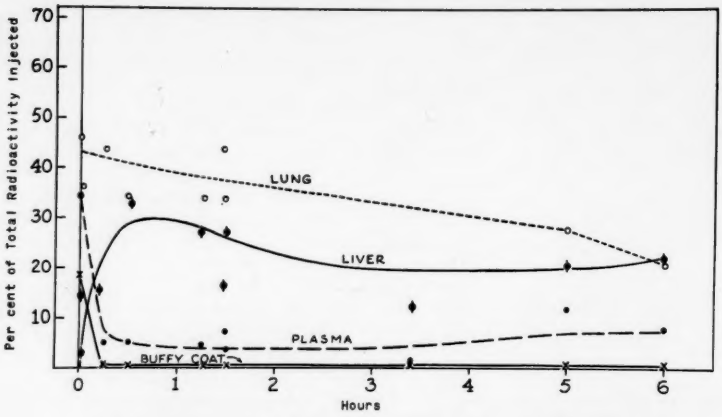



FIG. 2. DISTRIBUTION OF RADIOACTIVITY IN TOLERANT RABBITS
Each tolerant rabbit received an injection of a massive dose (6.0 mg.) of endotoxin.

muscle and intestine. The radioactivity of the tissues and injected chromium was determined as previously described in the scintillation counter after counting for 10 minutes (6). Total amount of circulating radioactivity in whole blood, plasma, buffy coat and red cells of each animal was calculated from the activity of the samples examined on the basis of an estimated total blood volume of 5 ml. per 100 grams of body weight. This figure for blood volume is based on our measurement in rabbits (6), as well as those of Courtice and Gunton (9), Kaplan (10), and Armin, Grant, Pels, and Reeve (11). Our attempts to measure the blood volume in mice with $Cr^{51}$ labeled red cells were unsuccessful and no reports were found in the literature. In rats, careful studies with $F^{59}$ and $P^{32}$ labeled red cells indicate that the blood volume is 4.59 plus or minus 0.57 ml. per 100 gram body weight (12).

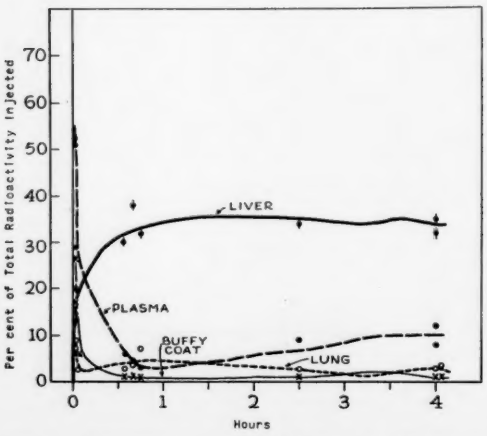



FIG. 3. DISTRIBUTION OF RADIOACTIVITY IN TOLERANT RABBITS WITH LOW PRECIPITIN TITERS
The average titer was 1:1. Each tolerant rabbit received an injection of a massive dose (6.0 mg.) of endotoxin.

6. *Precipitin tests.* Blood was collected from all rabbits by cardiac puncture on the day before the experiment and the serum used for determining the titer of antibody. One-half ml. of each serial dilution of serum was placed in a tube measuring 75 by 12 mm. and mixed with an equal volume of saline containing 1.0 mg. of endotoxin. The mixture was incubated at 37° C. for one to two hours and then refrigerated overnight. After centrifugation at 2,000 rpm for five minutes the tubes were examined macroscopically and the titer recorded as the highest dilution of serum accompanied by the formation of a gross precipitate.

For precipitin tests on the sera of mice, blood was collected on the day of the experiment by cardiac puncture of 6 to 12 mice in each group. These mice, which had received the same preparatory manipulations as the others in each group, were not subjected to tracer studies. Precipitin tests were performed on the sera of individual mice in capillary tubes measuring 1.5 to 2.0 mm. in diameter. Equal amounts of serum diluted serially and of a suspension of endotoxin (1 mg. per ml. of saline) were drawn into the tube and incubated at 37° C. for 48 hours and the zone of precipitate at the interface measured in millimeters.

## RESULTS

### *I. Tracer studies in rabbits (see Figures 1 through 5)*

### *A. Normals*

Endotoxin was carried in the plasma but not red cells immediately after intravenous injection.

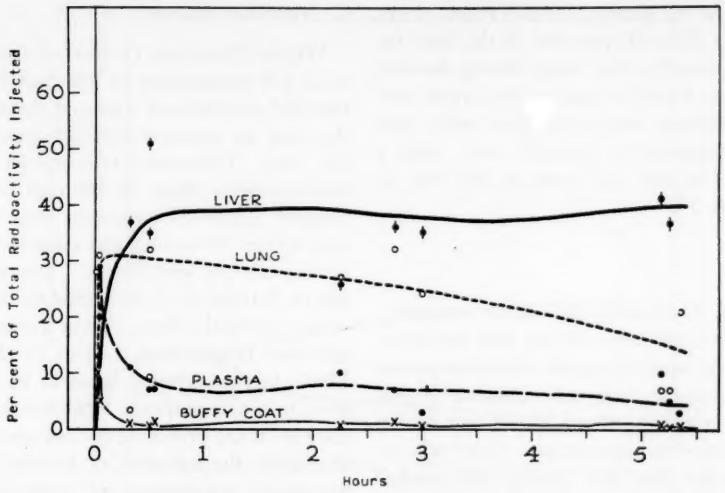



FIG. 4. DISTRIBUTION OF RADIOACTIVITY IN IRRADIATED TOLERANT RABBITS WITH HIGH PRECIPITIN TITERS

The average titer was 1:15. Each irradiated-tolerant rabbit received an injection of a massive dose (6.0 mg.) of radioactive endotoxin.

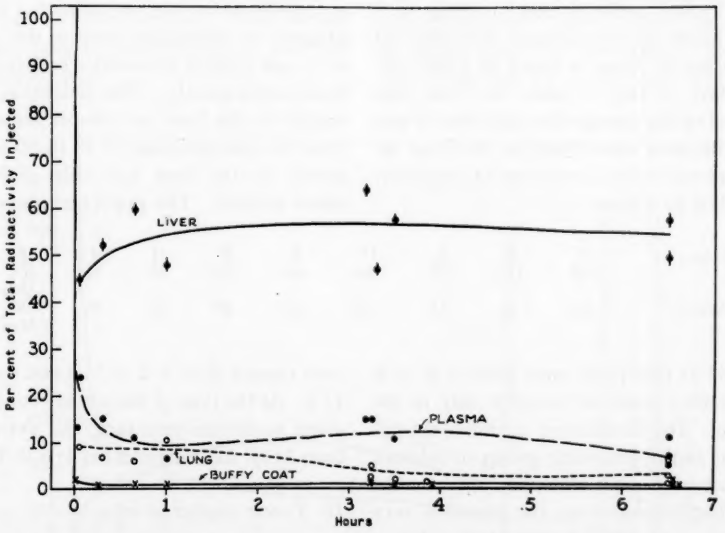



FIG. 5. DISTRIBUTION OF RADIOACTIVITY IN RABBITS WHOSE TOLERANCE WAS ALLOWED TO LAPSE

Each lapsed-tolerant rabbit received an injection of a massive dose (6.0 mg.) of radioactive endotoxin. Serum precipitin titers at height of tolerance, 1:16; after lapse of tolerance, 1:4.

Within an hour the plasma concentrations of endotoxin fell to 25 to 40 per cent of the total injected and remained in that range during the first four hours. As it left the plasma, the largest percentage of endotoxin went to the liver and a high concentration appeared on the buffy coat. Only a small per cent of the total went to the lung or spleen (Figure 1).

*B. Tolerant*

In less than 20 minutes, the plasma concentration of endotoxin fell below 10 per cent and never rose. The great majority of the endotoxin passed immediately into the lung and remained in that organ in concentrations above 30 per cent during most of the period of observation. Endotoxin accumulated in the liver less rapidly and reached concentrations of 20 to 30 per cent. An enormous amount of endotoxin (18.3 per cent) was found in the buffy coat immediately after injection but the concentration there fell rapidly afterwards (Figure 2). As a result of an accident, precipitin titers were not obtained on the sera of these animals.

In order to repeat the experiment in tolerant rabbits with known antibody titer, a group of 9 rabbits was given 12 intravenous injections of endotoxin during 32 days in doses of 0.001 mg. except on Days 3 and 5 when the dose was 0.0005 mg. and on the twenty-first day when it was 0.100 mg. Tolerance was evident by the fever indices of all animals upon completion of injections which had fallen as follows:

| Rabbit no. | A | B | C | D | E | F | H | I | J | L |
|---|---|---|---|---|---|---|---|---|---|---|
| Start | 138 | 130 | 88 | 104 | 104 | 50 | 56 | 76 | 102 | 40 |
| | | | | | | | | | Mean, | 88.8 |
| Completion | 58 | 40 | 28 | 30 | 40 | 40 | 28 | 36 | 38 | 28 |
| | | | | | | | | | Mean, | 36.8 |

In these rabbits precipitins were present in such low titer that they could be detected only in the undiluted sera. The distribution of endotoxin differs from that in the preceding group of tolerant animals in that only small amounts appear in the lung. The disappearance from the plasma is very rapid, however, with concentration falling below 10 per cent in 30 minutes. The massive uptake by buffy coat in concentrations reaching 27 per cent during the first few minutes is also found in these animals (Figure 3).

*C. Irradiated tolerant*

Within 30 minutes the level of circulating endotoxin fell permanently to less than 10 per cent of the total injected and appeared almost instantly in the lung in concentrations of approximately 30 per cent. Pulmonary radioactivity persisted in concentrations above 20 per cent for five hours. Hepatic uptake of endotoxin rose above 30 per cent within 30 minutes and remained at that level throughout the experiment. Despite the earlier use of X-irradiation, these rabbits developed high serum precipitin titers. Their average was 1:15, and they ranged from 1:8 to 1:32 (Figure 4). While this experiment failed in its primary purpose to arrest antibody formation by irradiation, the results are presented because they demonstrate in rabbits the influence of humoral antibody on pulmonary localization of endotoxin and thus amplify the result obtained in nonirradiated tolerant rabbits whose precipitin titer could not be measured.

*D. Lapsed tolerant*

Endotoxin was removed from the plasma almost as rapidly as in tolerant animals (Figure 5). It dropped to circulating levels in the average range of 11 per cent in 45 minutes and remained at low levels subsequently. The endotoxin was removed mainly by the liver and almost instantly appeared there in concentrations of 45 to 60 per cent. The uptake in the lung was only slightly increased above normal. The precipitin titers in these animals ranged from 1:2 to 1:8 and the average was 1:4. At the time of the last injection of endotoxin given to induce tolerance, the average titer had been 1:16 and ranged from 1:8 to 1:64.

*II. Tracer studies in mice*

*A. Normals*

The results are given in Figures 6 and 7. The rate of removal of a lethal dose from whole blood was the same for 0.5 mg. and 1.5 mg. of endotoxin.

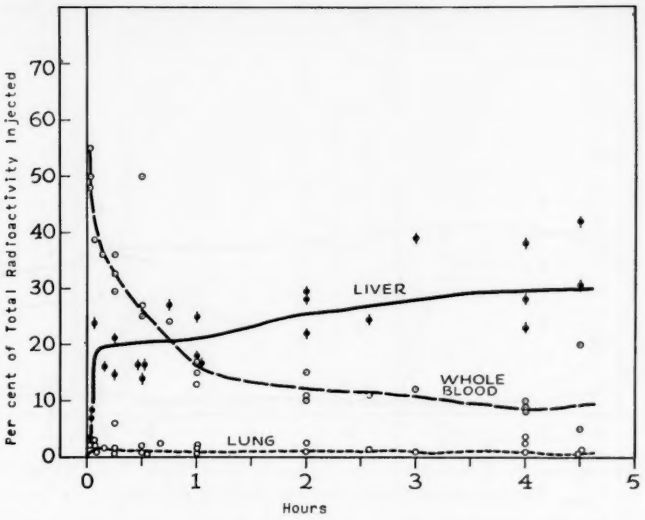



FIG. 6. DISTRIBUTION OF RADIOACTIVITY IN NORMAL MICE RECEIVING 1.5 MG. ENDOTOXIN

The circulating concentration remained above 20 per cent of the total injected during the first hour and most of it passed into the liver. The amount localizing in the lung seldom exceeded 2 per cent of the total radioactivity.

*B. Tolerant mice*

Tracer studies were performed in three groups of tolerant mice. In one group the precipitins in the serum were high (average, 1:32); in the second group low (1:2 or less); and in the third

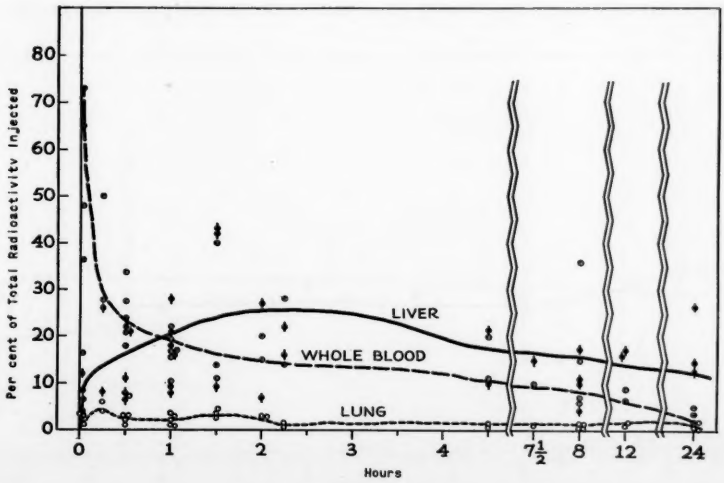



FIG. 7. DISTRIBUTION OF RADIOACTIVITY IN NORMAL MICE RECEIVING INJECTION OF 0.5 MG. ENDOTOXIN

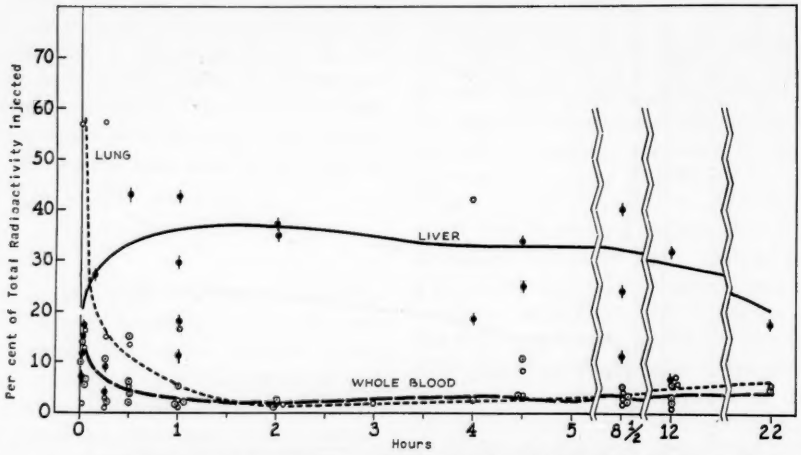



FIG. 8. DISTRIBUTION OF RADIOACTIVITY IN TOLERANT MICE WITH HIGH TITERS OF SERUM PRECIPITINS RECEIVING MASSIVE DOSES OF ENDOTOXIN

the precipitins were high (average, 1 : 32), but the animals received colloidal iron intravenously (2 mg. saccharated iron oxide) 12 hours before the tracer studies. The purpose of the colloidal iron was to block the reticuloendothelial system (13) and abolish tolerance; this was not successful.

*1) Tolerant mice with high titers of precipitins ($LD_{50}$, 2.22 mg.; normals, 0.055 mg.).* The composite results of two experiments are given in Figure 8. The removal curves resembled those of tolerant rabbits in the following respects: 1) immediate fall of blood level to less than 5 per cent; and 2) high *initial* uptake in lung in the majority of mice. Unlike tolerant rabbits, however, the endotoxin left the lung after an hour so that less than 10 per cent of that injected was found there beyond that time.

*2) Tolerant mice with low titers of precipitins ($LD_{50}$, 0.25 mg.; normals, 0.044).* These mice failed to develop a high titer of precipitins even though given the same schedule of injection of endotoxin as the preceding group. The results

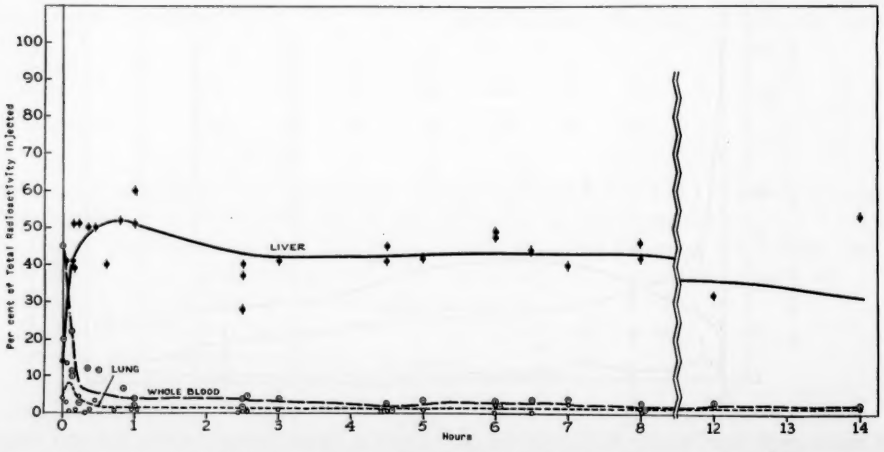



FIG. 9. DISTRIBUTION OF RADIOACTIVITY IN TOLERANT MICE WITH LOW TITERS OF SERUM PRECIPITINS (1 : 2) RECEIVING 1.5 MG. ENDOTOXIN

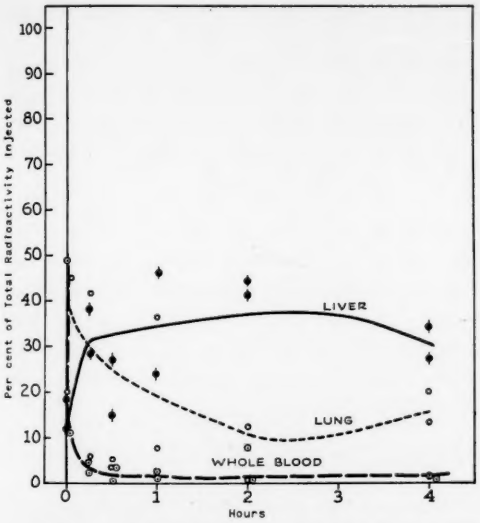



FIG. 10. EFFECT OF COLLOIDAL IRON ON DISTRIBUTION OF RADIOACTIVE ENDOTOXIN IN TWELVE TOLERANT MICE

Each had received intravenously 2 mg. of saccharated iron oxide 12 hours before intravenous injection of 1.5 mg. radioactive endotoxin.

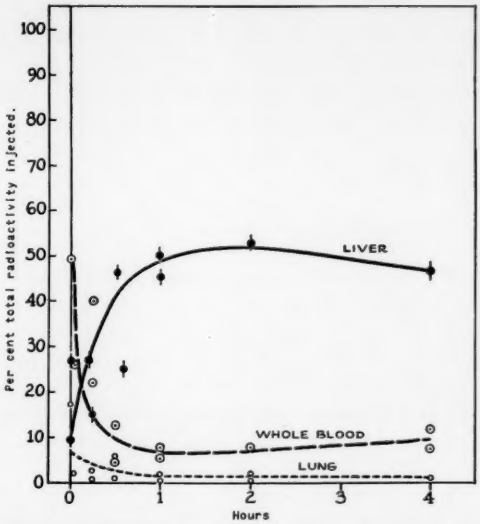



FIG. 11. DISTRIBUTION OF RADIOACTIVITY IN IRRADIATED-TOLERANT MICE RECEIVING 1.5 MG. ENDOTOXIN

Serum precipitin titers were less than 1:2. Titer in nonirradiated tolerant was 1:32.

are given in Figure 9. The distribution differs from that seen in tolerant mice with high titers in the following respects: 1) initial uptake in lung is low; 2) blood levels fall a little more slowly and not quite as low as in tolerant animals with high titers; and 3) uptake by the liver is faster and greater than in tolerant mice with high precipitin titers.

*3) Tolerant mice with high titers of precipitins pretreated with intravenous colloidal iron.*

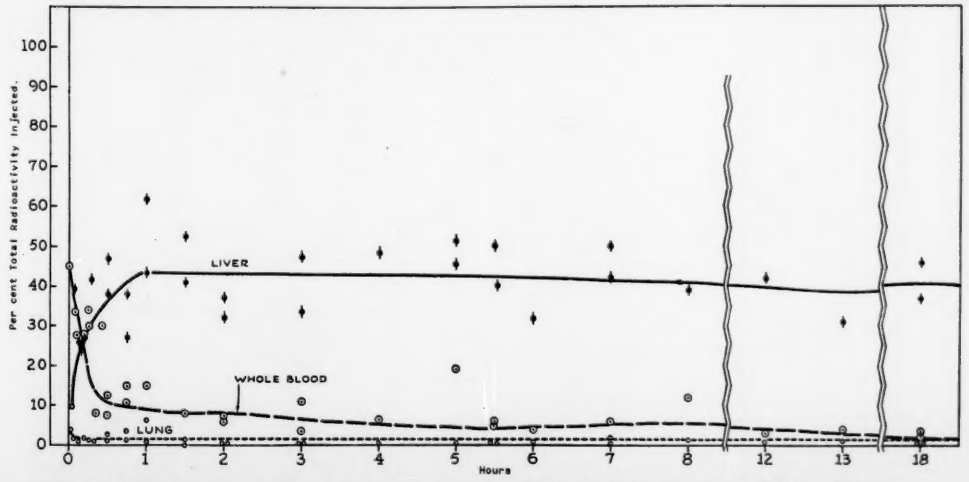



FIG. 12. DISTRIBUTION OF RADIOACTIVITY IN IRRADIATED-TOLERANT MICE RECEIVING 1.5 MG. ENDOTOXIN

Serum precipitins were absent in irradiated-tolerant animals and only 1:2 in the nonirradiated-tolerant.

The colloidal iron did not interfere with removal of endotoxin from the circulation (Figure 10) and did not reduce tolerance. In fact, tolerance was enhanced as shown by the following $LD_{50}$'s at the time of the distribution studies: 1) normals, 0.02; 2) tolerant, 1.22; 3) tolerant mice receiving colloidal iron, 3.5.

*C. Irradiated tolerant mice*

When each of the two groups of tolerant mice in the preceding experiment (one with high precipitin titers, and one with low titers) was made tolerant, a parallel group of irradiated mice was injected with endotoxin simultaneously and with identical dosages. The irradiation had been given in the form of 350 r (220 KV: 17 ma ½ Cu 1 Al filter at 50 cm.) at the rate of 59 r per mm. Injections were started in one group 24 hours after irradiation (Figure 11) and 3 hours after irradiation in the second group (Figure 12). Both groups of animals developed tolerance but the precipitin titers were very low (less than 1:2). In the first group, $LD_{50}$ equals 0.82 mg.; normals, 0.2 mg.; nonirradiated tolerant, 1.2 mg. In the second group, $LD_{50}$ equals 0.156 mg., normals, 0.086 mg.

1) The rate of removal from the blood was slower than in the corresponding tolerant mice, but faster and greater than the normal controls. Within one and one-half hours the level fell to 8 per cent (half that of normals).

2) There was almost no uptake by the lung.

3) Most of the endotoxin passed into the liver and greatly exceeded the uptake of nontolerant livers. It is especially noteworthy that the initial rapid disappearance from plasma into the lung was abolished in the group which failed to develop the high precipitin titer observed in its nonirradiated counterpart.

*D. Lapsed tolerant*

Two groups were studied:

*1)* Tolerance lapsed completely but antibodies persisted elevated to 1:16 to 1:32 (Figure 13). $LD_{50}$ at height of tolerance was 2.22 mg.; normals at that time, 0.55. $LD_{50}$ at time of distribution study was 0.089; normals, 0.071.

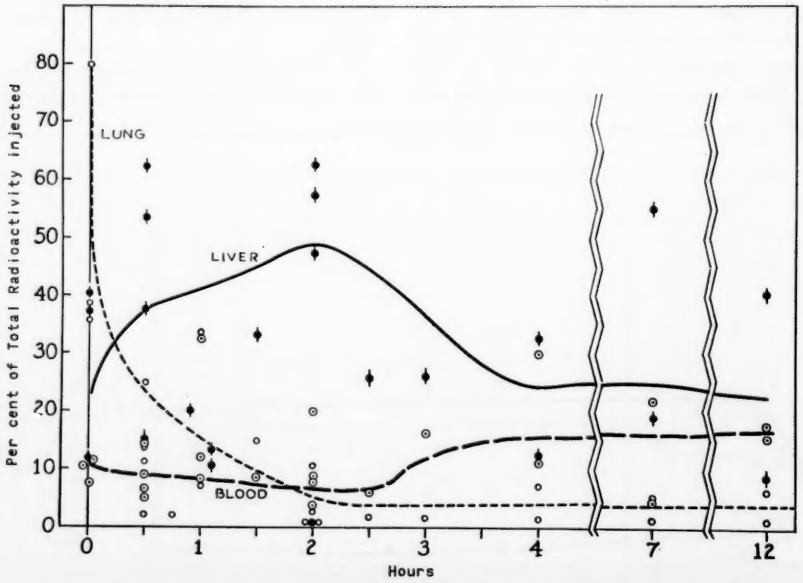



FIG. 13. DISTRIBUTION OF RADIOACTIVITY IN MICE WHOSE TOLERANCE HAD LAPSED
Each mouse received 0.5 mg. radioactive endotoxin intracardially. Serum precipitin titers equal 1:16 and 1:32.

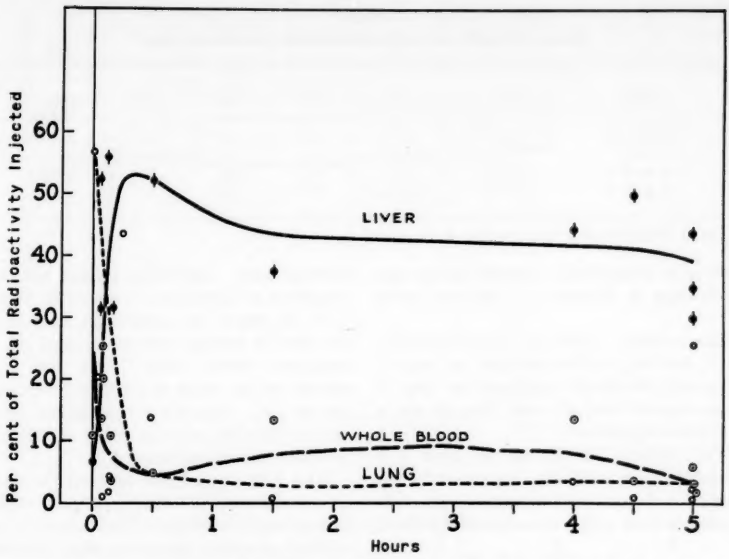



FIG. 14. DISTRIBUTION OF RADIOACTIVITY IN MICE WHOSE TOLERANCE HAD ONLY PARTIALLY LAPSED

Each animal received 1.5 mg. radioactive endotoxin intravenously. Serum precipitin titers equal 1:8 plus.

These animals showed an initial rapid removal by the lung, and the blood level then fell almost instantly to an average level of 10.8 per cent during the first two and one-half hours. Endotoxin also passed to the liver immediately and reached concentrations close to 50 per cent. But neither the lung nor the liver held the endotoxin, and it reappeared in the circulation. The mean concentration in the blood after three hours was 17 per cent, in contrast to the tolerant mice from the same group with high titers of precipitins (Figure 8) whose circulating level in the blood remained at a mean of 4 per cent (S.E. of tolerant after three hours, 0.882; S.E. lapsed tolerant after three hours, 1.72. Standard error of difference, 3.02; t, 4.3; $p < 0.01$).

*2*) Tolerance lapsed only partially and antibody titer remained elevated. $LD_{80}$ at height of tolerance was 1.22 mg.; normal, 0.2. $LD_{50}$ at time of distribution study was 0.263; normal, 0.122 (Figure 14).

The mice that retained partial tolerance did not relinquish their initial high concentrations of endotoxin in the liver, so that circulating blood levels remained at a mean of 5.2 per cent after three hours.

## EXPERIMENTAL

*Small sublethal doses of endotoxin*

The materials and methods were the same as those described above, except as noted below.

Rabbits were divided into three groups. One group was made tolerant to the pyrogenic action by injecting

TABLE IV

*Fever indices before and after development of tolerance in rabbits receiving small doses of endotoxin*

| | Schedule I | | | Schedule II | |
|---|---|---|---|---|---|
| Rabbit | Before | After | Rabbit | Before | After |
| A 6 | 80 | 20 | A 13 | 106 | 56 |
| O 6 | 112 | 48 | B 13 | 126 | 46 |
| H 6 | 100 | 78 | C 13 | 114 | 38 |
| G 6 | 114 | 102 | D 13 | 96 | 16 |
| C 6 | 90 | 68 | E 13 | 84 | 60 |
| B 6 | 104 | 78 | H 13 | 66 | 36 |
| M 6 | 112 | 60 | K 13 | 124 | 74 |
| D 6 | 142 | 92 | L 13 | 74 | 50 |
| E | 110 | 72 | M 13 | 126 | 34 |
| | | | P 13 | 90 | 36 |
| Mean index | 110.6 | 68.6 | | 100.6 | 44.6 |

TABLE V

*Serum precipitin titer against endotoxin in tolerant mice**

| Mouse no. | Undil. | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | Serum control No antigen |
|---|---|---|---|---|---|---|---|---|
| 1 | ++++ | ++ | ++ | + | − | − | − | − |
| 2 | ++ | + | + | − | − | − | − | − |
| 3 | ++++ | +++ | ++ | + | ± | + | + | − |
| 4 | ++++ | +++ | ++ | + | + | − | − | − |

* The amount of precipitate is recorded as + to ++++.

endotoxin according to Schedule I; a second group was made tolerant according to Schedule II; and one group was untreated.

Schedule I (nine rabbits): 0.025 mg. intraperitoneally on Days 1 and 3; 0.05 mg. intraperitoneally on Day 5; 0.05 mg. intravenously thereafter beginning on Day 9 and continuing at regular intervals until Day 41 for a total of 17 intravenous injections.

Schedule II (ten rabbits): 0.0005 mg. on Days 1, 2 and 4 intravenously. Then 0.001 mg. intravenously beginning on Day 10 and continued at regular intervals until Day 58 when a total of 17 intravenous injections was given.

In both Groups I and II, the existence of tolerance to the injection of endotoxin was demonstrated by the characteristically reduced febrile response, and the better physical condition of each animal when compared to non-tolerant ones. The fever indices before and after development of tolerance are shown in Table IV.

On the day of the experiment, each of 21 normal rabbits and 19 tolerant rabbits received 0.1 mg. of *E. coli* endotoxin labeled with $Cr^{51}Cl_3$ and possessing radioactivity in the range of 5,440 to 7,000 counts per second per mg. (7). Animals were sacrificed immediately and at various intervals after injection of endotoxin by cardiac puncture and exsanguination.

Mice were divided into two groups. One group served as controls and the other was made tolerant by the intraperitoneal injection of endotoxin according to the schedule described above for mice receiving lethal doses.

The $LD_{50}$ of tolerant mice on the day of the distribution study was 0.23 mg. endotoxin and that of the normal controls was 0.03 mg. endotoxin. The tolerant mice were then eight times more resistant than normals to the

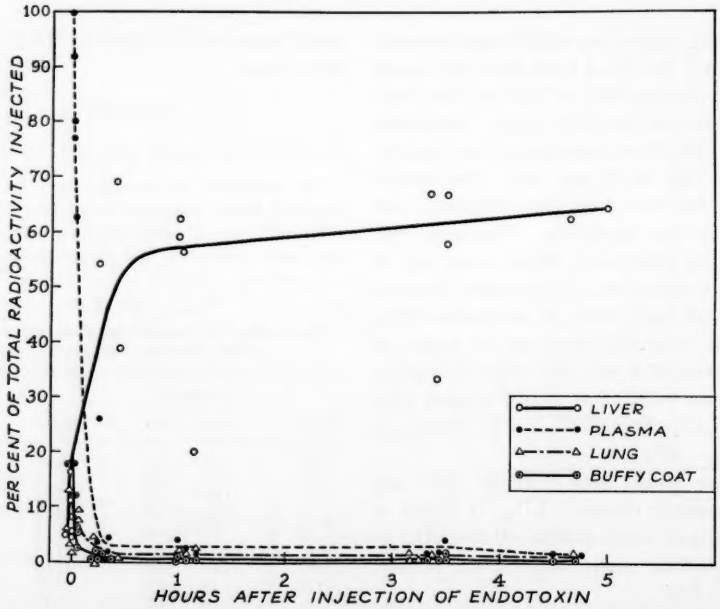



FIG. 15A. DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF SUBLETHAL DOSES OF ENDOTOXIN IN TWENTY-ONE NORMAL RABBITS

Each rabbit received 0.1 mg. *E. coli* endotoxin labeled with $Cr^{51}Cl_3$.

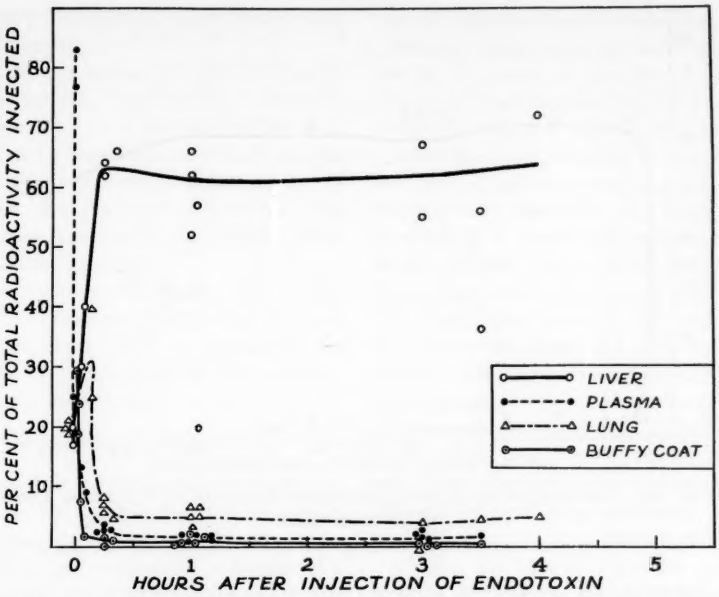



FIG. 15B. DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF SUBLETHAL DOSES OF ENDOTOXIN IN NINETEEN TOLERANT RABBITS

Each rabbit received 0.1 mg. *E. coli* endotoxin labeled with $Cr^{51}Cl_3$.

lethal effect of endotoxin. Cardiac blood was obtained in four mice from the group made tolerant and serum precipitin titers were determined as described in capillary tubes. Equal amounts of serum and endotoxin were drawn into the tubes which were then incubated at 37° C. for three hours. The results are shown in Table V. Each animal was given intravenously 0.01 mg. of endotoxin labeled with $Na_2Cr^{51}O_4$ and sacrificed at the various in-

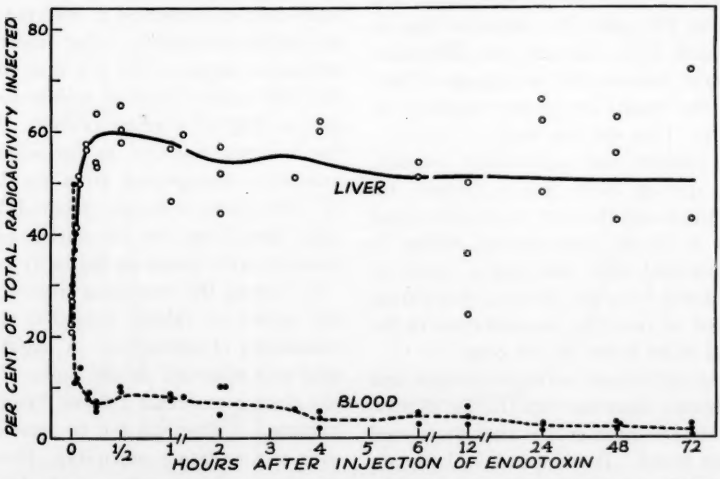



FIG. 16A. DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF SUBLETHAL DOSES IN NONTOLERANT MICE

Each animal received intravenously 0.01 mg. *E. coli* endotoxin labeled with $Na_2Cr^{51}O_4$.

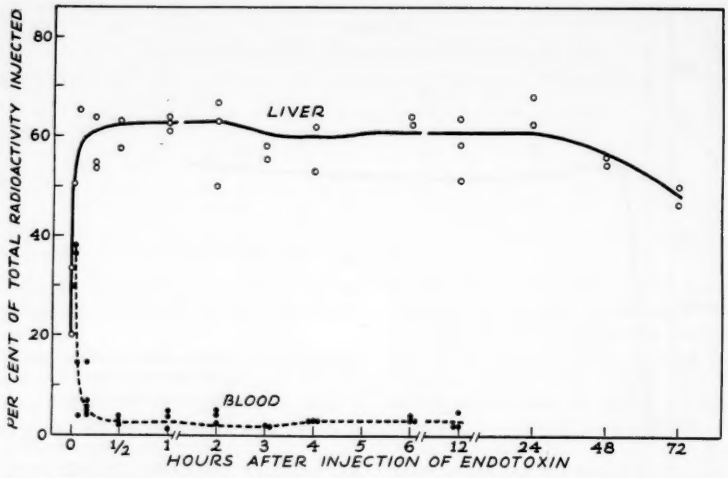



FIG. 16B. DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF SUBLETHAL DOSES IN TOLERANT MICE

Each animal received intravenously 0.01 mg. *E. coli* endotoxin labeled with $Na_2Cr^{51}O_4$.

tervals designated in Figure 16A and 16B. Each mg. of injected radioactive endotoxin produced 1,270 counts per second in the scintillation counter.

## RESULTS

The distribution curves in both rabbits and mice given sublethal doses are very similar and can be described together because they elucidate the same four points. The results for rabbits are given in Figures 15A and 15B; those for mice in Figures 16A and 16B. Because no differences were encountered between the two groups of tolerant rabbits, the results are plotted together on the same figure. They disclose that:

1. In both tolerant and nontolerant animals, regardless of species, there was a massive removal of endotoxin into the liver in amounts equal approximately to 60 per cent or more within 15 minutes. Associated with this was a rapid removal of endotoxin from the blood so that during the same period of time the concentration in the circulation had fallen below 10 per cent.

2. The great differences between tolerant and nontolerant animals observed with the use of massive doses (60 to 150 times greater than those used here) were not found. It was possible, however, to detect slight but consistent differences in the two groups. In tolerant mice, blood concentration fell below 5 per cent within 15 minutes and remained there, while in nontolerant mice the blood concentration remained between 5 and 10 per cent for four hours. Distribution curves for the liver also suggested that slightly higher percentages of endotoxin appear there in tolerant animals (both rabbits and mice) than in nontolerant animals.

3. A heavy distribution of endotoxin in the buffy coat was observed in both normal and tolerant rabbits immediately after injection. Concentrations as high as 17.7 per cent were present in the buffy coat of normal rabbits and of 29.4 per cent in that of tolerant rabbits. After the first few minutes, however, as the polymorphonuclear leukocytes disappeared from the blood (Figure 17), these cells seemingly removed their endotoxin with them from the circulation and only small amounts were found in the buffy coat thereafter.

4. Among the remaining tissues, only the lung and spleen of rabbits exhibited significant concentrations of endotoxin. A slightly greater uptake was observed in the lungs of tolerant rabbits than nontolerant rabbits during the first 15 minutes. This could not be related to the presence of circulating precipitins, however, because these were absent in the group of tolerant rabbits used here. In mice, even though precipitins were demonstrated, the titer was apparently too low to

effect pulmonary localization with these small amounts of endotoxin.

The uptake by the spleen in mice was barely detectable. In rabbits, however, the concentrations varied from 0.5 to 3 per cent of the total endotoxin injected. Based on the relative weight of the spleen, this represents a concentration of 20 to 80 times. No important differences were discerned between the uptake by spleens from normal and tolerant animals.

Only traces of endotoxin, if any, were found in the urine or intestinal contents so that removal from the blood could be accounted for almost entirely by localization in the reticuloendothelial system in the liver.

## DISCUSSION

Susceptibility to the lethal action of endotoxin in mice and to its pyrogenic effect in rabbits seemed directly related to the speed and permanence with which massive doses of endotoxin disappeared from the blood. All tolerant animals removed massive doses of endotoxin from the blood in much less time than did normal animals. Unless such massive amounts of endotoxin were injected, however, this capacity for accelerated removal in tolerant animals was not apparent; instead both normal and tolerant animals rapidly cleared sublethal doses of endotoxin from the circulation at approximately equal rates. The following discussion of the altered distribution in tolerant animals is therefore concerned only with the results obtained with massive doses.

Upon the swift removal of massive doses from the blood of tolerant mice, endotoxin passed into the liver and sometimes the lung. The uptake by the lung in rabbits and mice depended on the serum antibody titer. With precipitin titers in the average range of 1:8 or higher, high pulmonary localization occurred after injection of massive doses; with titers of 1:2 or less, it never occurred. The high pulmonary uptake and the high antibody titer were not essential, however, for tolerance or speedy removal. Thus, tolerance was induced and antibody formation suppressed in mice given irradiation just before the start of injections for producing tolerance; and nonirradiated mice and rabbits developed tolerance after the usual schedule of injections without always forming high titers of circulating antibody. In these two groups of tolerant mice (irradiated tolerant and normal tolerant with low precipitins), endotoxin was removed from the blood more rapidly than normally but not as fast as in those mice with antibody titers of 1:8 or higher.

Although antibody thus contributes to the swift

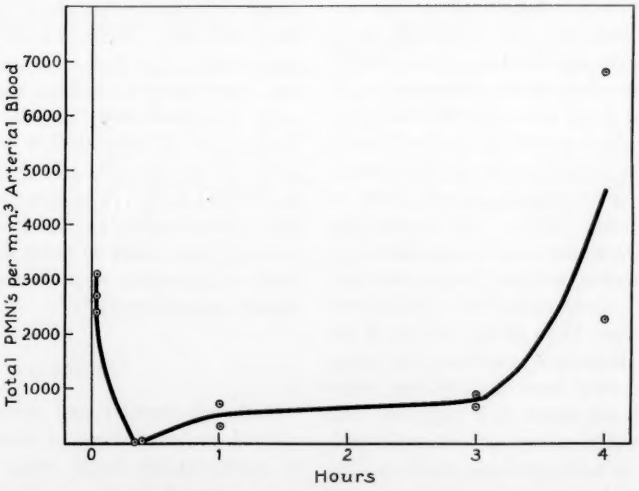



FIG. 17. POLYMORPHONUCLEAR LEUKOCYTE (PMN) COUNTS IN NONTOLERANT RABBITS RECEIVING 0.1 MG. *E. COLI* ENDOTOXIN LABELED WITH $Cr^{51}Cl_3$

removal into the lung, high titers of antibody cannot by themselves provide protection in mice. A demonstration of this was observed in mice whose tolerance disappeared despite the persistence of circulating antibody at a titer of 1:16 or above. In these animals circulating endotoxin rapidly disappeared into the lung; after two hours, however, pulmonary concentrations fell almost to zero and circulating endotoxin reappeared in high concentrations. It would appear, therefore, that circulating antibody may contribute to tolerance in mice by inducing rapid but temporary pulmonary removal of endotoxin. Tolerance only became evident, however, when endotoxin from the lung localized in the liver at a rate which kept the circulating level from rising. Removal of endotoxin by the liver in tolerant mice was probably the principal factor responsible for maintaining a low circulating level of endotoxin. The low pulmonary uptake in tolerant animals lacking high titers of precipitins was compensated for by a high rapid initial uptake by the liver. Unlike mice, the pulmonary localization of endotoxin in high concentration persisted in tolerant rabbits with high titers of precipitins. It is possible, therefore, that in rabbits the presence of antibody provides a less transient protective action.

The relationship between precipitin titer and the immediate pulmonary localization after intravenous injection of lethal doses suggests that precipitins combine instantly with endotoxin as it enters the circulation and thereby forms precipitates which are filtered out by the pulmonary capillaries. Precipitins could also alter the surface of endotoxin particles and render them more suitable for ingestion by the numerous and actively phagocytic pulmonary macrophages (septal cells of Maximow and Bloom [14]). The speed with which endotoxin leaves the lung in mice makes the process of precipitation without phagocytosis appear to be a more likely possibility. Pulmonary levels may drop from 50 to 60 per cent to 3 per cent in one hour. Because it is unlikely that phagocytosed particles could leave the cells and reappear so rapidly in the blood, it is suggested that the pulmonary capillaries trap the endotoxin-antibody precipitates in mice and later capillary dilation enables them to be washed out into the general circulation where they can break up into smaller particles. In rabbits, on the other hand, the persistence of radioactivity in the lung favors the possibility that the pulmonary macrophages may ingest precipitated particles.

The tolerance which occurs in the absence of circulating antibody is accompanied by a marked increase in hepatic localization of endotoxin after injection of lethal doses. This would suggest that the Kupffer cells become more active individually in removing massive lethal doses of endotoxin, and that their ability to do so may represent a cellular immunity characterized by an increased resistance of the individual reticuloendothelial cells to the damaging action of the endotoxin. That such damage occurs is indicated by: 1) the work of Biozzi, Benacerraf, and Halpern (15) who found that the endotoxin of *Salmonella typhosa* suppressed the ability of hepatic reticuloendothelial cells to ingest carbon particles; and 2) the observation that the first dose of endotoxin given to prepare rabbits for the Shwartzman reaction cut in half the ability of the liver to remove a second dose of endotoxin injected into the circulation during the provocative stage (16).

Another possibility is that tolerance leads to a proliferation of Kupffer cells so that their increased number can then cope with larger quantities of circulating endotoxin. Either mechanism would hardly be consistent, however, with the failure of the colloidal iron used in these experiments to block hepatic uptake in mice and reduce their tolerance. While it is likely that the dosage of colloidal iron and other experimental conditions may account for these negative results, they raise the possibility that the increased hepatic localization of radioactivity may be related to other factors than the altered activity of reticuloendothelial cells. It is conceivable, for example, that tolerant animals differ from nontolerant animals in their ability to resist the profound alterations in splanchnic blood flow which influence the hepatic circulation (17).

## SUMMARY

Although normal and tolerant animals both cleared sublethal doses of endotoxin rapidly and at approximately equal rates, a great difference was observed between tolerant and nontolerant animals in the hepatic localization and plasma clearance of massive doses of endotoxin. In tol-

erant animals massive doses of radioactive endotoxin were removed much more rapidly from the circulating blood and more of it was deposited in the liver and leukocytes than in the nontolerant animal. If circulating antibody (precipitin) was present in high titer, the endotoxin was removed from the circulation still more rapidly because of an enormous and instantaneous deposition in the lung. In mice this pulmonary deposition was only transient; but in rabbits the high pulmonary localization lasted for several hours.

These results indicate that tolerance to lethal doses of endotoxin depends on speedy and permanent removal of circulating endotoxin into hepatic and other cells. Although not essential for tolerance, circulating antibody may contribute to it by inducing rapid but temporary pulmonary removal which by itself cannot provide protection.

## REFERENCES


1. Morgan, H. R. Tolerance to the toxic action of somatic antigens of enteric bacteria. J. Immunol. 1948, **59**, 129.
2. Morgan, H. R. Resistance to the action of the endotoxins of enteric bacilli in man. J. clin. Invest. 1948, **27**, 706.
3. Beeson, P. B. Tolerance to bacterial pyrogens. I. Factors influencing its development. J. exp. Med. 1947, **86**, 29.
4. Beeson, P. B. Development of tolerance to typhoid bacterial pyrogen and its abolition by reticuloendothelial blockade. Proc. Soc. exp. Biol. (N. Y.) 1946, **61**, 248.
5. Beeson, P. B. Effect of reticulo-endothelial blockade on immunity to Shwartzman phenomenon. Proc. Soc. exp. Biol. (N. Y.) 1947, **64**, 146.
6. Braude, A. I., Carey, F. J., and Zalesky, M. Studies with radioactive endotoxin. II. Correlation of physiologic effects with distribution of radioactivity in rabbits injected with lethal doses of *E. coli* endotoxin labelled with radioactive sodium chromate. J. clin. Invest. 1955, **34**, 858.
7. Braude, A. I., Carey, F. J., Sutherland, D., and Zalesky, M. Studies with radioactive endotoxin. I. The use of $Cr^{51}$ to label endotoxin of *Escherichia coli*. J. clin. Invest. 1955, **34**, 850.
8. Reed, L. J., and Muench, H. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. 1938, **27**, 493.
9. Courtice, F. C., and Gunton, R. W. The determination of blood volume by the carbon monoxide and dye (T-1824) methods in rabbits. J. Physiol. (Lond.) 1949, **108**, 405.
10. Kaplan, H. M. The rabbit in experimental physiology. New York, Scholar's Library, 1956.
11. Armin, J., Grant, R. T., Pels, H., and Reeve, E. B. The plasma, cell, and blood volumes of albino rabbits as estimated by the dye (T-1824) and $^{32}P$ marked cell methods. J. Physiol. (Lond.) 1952, **116**, 59.
12. Berlin, N. I., Huff, R. L., Van Dyke, D. C., and Hennessy, T. G. The blood volume of the adult rat, as determined with $Fe^{59}$ and $P^{32}$ labelled red cells. Proc. Soc. exp. Biol. (N. Y.) 1949, **71**, 176.
13. Smith, R. T., Lewis, T., and Good, R. A. Generalized Shwartzman reaction. V. Intravenous injection of colloidal iron or carbon on response of rabbits to meningococcal toxin. Proc. Soc. exp. Biol. (N. Y.) 1953, **82**, 712.
14. Maximow, A. A., and Bloom, W. A Textbook of Histology. Philadelphia, W. B. Saunders Co., 1930.
15. Biozzi, G., Benacerraf, B., and Halpern, B. N. The effect of *Salm. typhi* and its endotoxin on the phagocytic activity of the reticulo-endothelial system in mice. Brit. J. exp. Path. 1955, **36**, 226.
16. Smith, R. T., Braude, A. I., and Carey, F. J. The distribution of $Cr^{51}$ labeled *E. coli* endotoxin in the generalized Shwartzman reaction. J. clin. Invest. 1957, **36**, 695.
17. Weil, M. H., MacLean, L. D., Visscher, M. B., and Spink, W. W. Studies on the circulatory changes in the dog produced by endotoxin from gram-negative microorganisms. J. clin. Invest. 1956, **35**, 1191.

# THE EFFECT OF VARIATIONS IN SOLUTE EXCRETION AND VASOPRESSIN DOSAGE ON THE EXCRETION OF WATER IN THE DOG

By JACK ORLOFF, HENRY N. WAGNER, Jr., and DOUGLAS G. DAVIDSON

*(From the Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, National Institutes of Health, Bethesda, Md.)*

(Submitted for publication September 23, 1957; accepted November 21, 1957)

It has been known for some time that osmotic diuretics may modify the effect of vasopressin on urine concentration (1, 2). However, it has been generally assumed that urine will remain hypertonic to plasma if sufficient hormone is administered irrespective of the extent of the solute diuresis. This conclusion was based on the thesis that the major action of vasopressin is to promote the elaboration of a hypertonic urine presumably by a process involving active water reabsorption. Smith (3) and Wesson and Anslow (4), on the other hand, have suggested that vasopressin increases permeability of the tubule membrane to water in the distal segment, permitting osmotic equilibration in this area, and thereby diminishing the volume of isosmotic fluid delivered to a hypothetical concentrating site in the collecting duct.

The experiments to be reported were designed to reexamine in a quantitative fashion the effect of solute excretion on the renal response to purified arginine vasopressin. The data have been interpreted in the light of present information concerning the probable mode and sites of action of the hormone.

## METHODS AND MATERIAL

Experiments were performed on trained, unanesthetized female dogs weighing approximately 20 Kg. each. Preliminary studies were performed on animals which had undergone section of the supraoptico-hypophyseal tracts and pituitary stalk. Despite persistent polyuria and polydipsia it was impossible to rule out the presence of residual antidiuretic hormone in these animals. The infusion of hypertonic saline during water diuresis resulted in a gradual decrease in free water clearance ($C_{H_2O}$, see below), albeit at a lesser rate than in normal dogs; and further, early morning urine was invariably hypertonic to plasma. Consequently it was concluded that these preparations afforded no significant advantage over normal dogs for the purpose of these studies.

All experiments were performed following a 12 to 16 hour fast. Water was allowed *ad lib.* when necessary. Dogs were loosely restrained and maintained in the supine position throughout the experimental procedure. Extraneous stimuli were reduced to a minimum to insure adequate suppression of antidiuretic hormone release in hydrated animals. Bladder urine was collected by means of a soft indwelling rubber catheter. At the beginning of each study the dogs were given a quantity of water by stomach tube equal to 3 per cent of their body weight. Thereafter this positive water balance was maintained by administering 2.5 per cent glucose in water intravenously at a rate sufficient to maintain plasma osmolality relatively constant. Solute diuresis was produced by intravenous administration of an isotonic solution of mannitol into the opposite limb.

The clearance of inulin or creatinine was used as a measure of glomerular filtration rate. Creatinine was determined by the method of Kennedy, Hilton, and Berliner (5) and inulin by the method of Walser, Davidson, and Orloff (6). Urine and plasma osmolality were determined cryoscopically using the instrument developed by Bowman, Trantham, and Caulfield (7). All solutions were administered intravenously using a constant infusion pump.

Purified arginine vasopressin (lot no. AVN-5) was provided through the courtesy of Dr. V. du Vigneaud and contained no oxytocin by amino acid analysis. The potency of vasopressin had been assayed on the basis of its pressor activity. Stability of the hormone in aqueous solution was insured by maintaining the pH of all infusions in which the hormone was incorporated at approximately 5.0. In those studies in which large doses were required, commercial vasopressin (Pitressin®, Parke-Davis) was administered. A sufficient period of continuous vasopressin administration was allowed prior to the onset of all studies to insure stability of effect. This period of equilibration, designed to establish an approximately steady state, varied from 20 to 160 minutes and was inversely related to the magnitude of the dose.

Since changes in glomerular filtration rate as well as variations in the dosage of vasopressin and solute excretion may influence urinary water excretion, all dogs were maintained on a relatively constant high protein food intake. Constancy of both the diet and the state of hydration minimized daily variations in filtration rate in individual animals. Consequently it was possible to consider solute excretion and the dosage of vasopressin as the major variables in these studies, and changes in glomerular filtration rate of minor significance in this regard.

## RESULTS

### *I. The relationship between urine flow and solute excretion during suppression of antidiuretic hormone (ADH) release and in the presence of maximal vasopressin activity*

The relationship between urine flow and solute excretion during suppression of endogenous antidiuretic hormone release was examined in three normal dogs. These data were required to afford a basis for the subsequent comparison of the effects of graded doses of vasopressin on the relationship. A representative experiment is presented in Table I. The results of all studies are depicted in Figure 1 (upper curve). Each point on the graph represents an individual collection period. Urine flow is plotted on the ordinate and osmolar clearance ($C_{osm}$) on the abscissa. Solute-free water clearance ($C_{H_2O}$) is the vertical distance between the diagonal line (isosmotic parameter) and the experimentally derived point.[1] It is apparent that in the "absence" of endogenous ADH, solute-free water clearance rose progressively as the excretion of solute increased. Although a concomitant rise in sodium excretion was noted uniformly, it was not possible to distinguish between the effects of mannitol and sodium excretion on the results.

TABLE I

*Effect of solute excretion on free water clearance during suppression of endogenous antidiuretic hormone release (Dog L., 16 Kg.)*

| Time | Urine flow | $C_{osm}$ | $C_{H_2O}$ | $C_{Cr}$ | $P_{osm}$ |
|---|---|---|---|---|---|
| *min.* | *ml./min.* | | | | *μosm/Kg. $H_2O$* |
| 0 | 480 ml. $H_2O$ administered by stomach tube | | | | |
| 10 | Infuse 12 mg./min. of creatinine in 10 ml./min. of 2.5% dextrose in water | | | | |
| 104–124 | 7.4 | 2.2 | 5.2 | 66 | 270 |
| −148 | 7.1 | 2.0 | 5.1 | 62 | 270 |
| −164 | 7.6 | 2.0 | 5.6 | 62 | 268 |
| 166 | Begin additional infusion of 5% mannitol in $H_2O$ at 14 ml./min. | | | | |
| 164–200 | 10.9 | 4.1 | 6.9 | 59 | 268 |
| 201 | Increase dextrose infusion to 14 ml./min. Decrease mannitol to 5 ml./min. | | | | |
| 216 | Decrease dextrose infusion to 10.5 ml./min. | | | | |
| 200–230 | 12.8 | 6.1 | 6.7 | 56 | 270 |
| −260 | 12.6 | 6.3 | 6.4 | 58 | 269 |
| −290 | 13.4 | 6.7 | 6.7 | 60 | 268 |
| −320 | 14.8 | 7.5 | 7.3 | 66 | 269 |
| −380 | 17.6 | 9.0 | 8.6 | 76 | 273 |
| 380 | Increase dextrose infusion to 14 ml./min. | | | | |
| 380–406 | 17.5 | 9.0 | 8.5 | 79 | 272 |
| −424 | 18.4 | 9.1 | 9.1 | 82 | 271 |

The effect of 50 mU per Kg. per hour of purified arginine vasopressin administered to the three dogs during combined mannitol and water diuresis is depicted in the lower curve (Figure 1). Under these circumstances urine remained hypertonic to plasma throughout the entire range of solute excretion examined. If sufficient time were allowed to achieve a maximal or steady state effect (60 to 90 minutes at this dosage of vaspressin) 5 mU per Kg. per hour of purified hormone was sufficient in some studies to produce effects indistinguishable from those noted with the larger dose. Consequently it may be concluded that the relationship depicted in the lower curve represents the effect of "maximal" ADH activity for the range of solute excretion examined.

### *II. The relationship between urine flow and solute excretion during administration of submaximal doses of vasopressin*

Fifteen studies in the three dogs were performed during which vasopressin was adminis-

[1] According to Wesson and Anslow (4), hypotonic urine may be considered as consisting of two hypothetical moieties: 1) an isosmotic portion (referred to as osmolar clearance [$C_{osm}$] and equal to $U_{osm}$ V/$P_{osm}$, where $U_{osm}$ and $P_{osm}$ refer to urine and plasma osmolality, respectively, and V to urine flow in ml. per minute), and 2) an additional amount of solute-free water equal in volume to that which would have to be added to the isosmotic moiety to reduce its concentration to that observed (referred to as $C_{H_2O}$). Thus, V equals $C_{osm}$ plus $C_{H_2O}$. Hypertonic urine, on the other hand, is depicted as being made up of an isosmotic portion ($C_{osm}$) less that amount of water which would have to be abstracted from it to produce the observed hypertonicity (referred to as $T^c_{H_2O}$). Thus, $T^c_{H_2O}$ equals $C_{osm}$ minus V. Although this has proved to be a useful concept, it should not be construed as necessarily affording any insight into the mechanism of urinary dilution and concentration. Unless so recognized, the calculations may be misleading. Thus, during water diuresis, $C_{H_2O}$ should not be visualized as depicting the total amount of water freed by sodium chloride abstraction from isosmotic urine but may well be the algebraic sum of water freed in this manner minus that lost by simple diffusion out of the renal tubule. The observed $C_{H_2O}$ will therefore be equal to the water freed minus the sum of that diffusing out at the same site or elsewhere in the diluting segment and that abstracted in the concentrating site. Similar considerations apply to the calculation of $T^c_{H_2O}$ since this too may represent the resultant of a number of processes, *i.e.*, water freeing, back-diffusion, and a terminal process which results in hypertonicity.

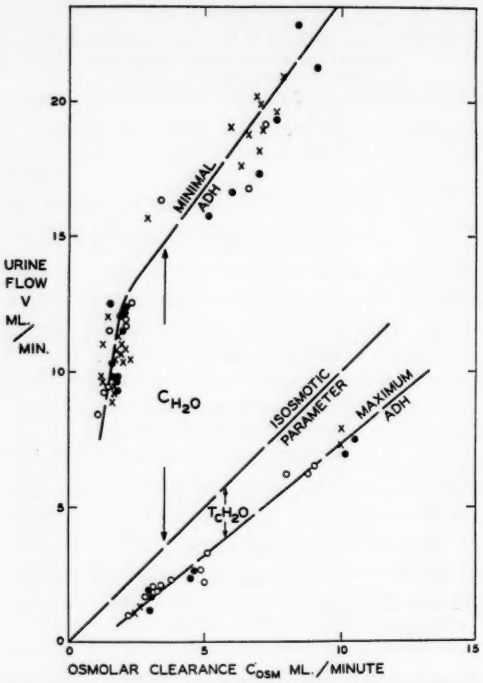



FIG. 1. THE RELATIONSHIP BETWEEN URINE FLOW AND SOLUTE EXCRETION IN THE ABSENCE OF ADH AND DURING THE ADMINISTRATION OF "MAXIMAL" DOSES OF VASOPRESSIN

tered in doses of either 0.5, 1.0, 1.5 or 4.0 mU per Kg. per hour. The results from all experiments are graphically illustrated in Figure 2. Each symbol represents an individual collection period. Although as noted above it was not always possible to distinguish between the effects of 5 and 50 mU per Kg. per hour of vasopressin once a steady state had been achieved, at the intermediate dosages marked differences in response were noted. In the range of 0.5 to 1.5 mU per Kg. per hour increased rates of vasopressin clearly resulted in lower rates of solute-free water clearance.[2] Although data in all studies were qualitatively similar, in that $C_{H_2O}$ diminished at the higher rates of hormone administration in single experiments, the absolute values of $C_{H_2O}$ at comparable $C_{osm}$ were sufficiently different in some studies at the same vasopressin dosage to preclude any precise quantitative comparison between the dogs. This is to be expected in any biological study of this nature, but is noted in order to emphasize the difficulty of predicting with any degree of precision the response in dogs to a specific dosage of vasopressin insofar as $C_{H_2O}$ is concerned. It should be noted that urine remained hypotonic to plasma during the administration of either 0.5 or 1.0 mU per Kg. per hour of vasopressin. Urine hypertonicity did not supervene at these dosages at the lowest rates of solute excretion despite administration of the hormone for as long as 160 minutes, a time presumably sufficient to achieve a steady state. When 1.5 mU per Kg. per hour (4 mU per Kg. per hour in one study) of arginine vasopressin was administered, hypertonic urine was elaborated only at the lower rates of solute excretion. At higher rates of solute excretion and despite the continuing administration of the same dosage of vasopressin, urine became hypotonic and $C_{H_2O}$ rose. The change in urine concentration from hypertonic to hypotonic was not observed at higher

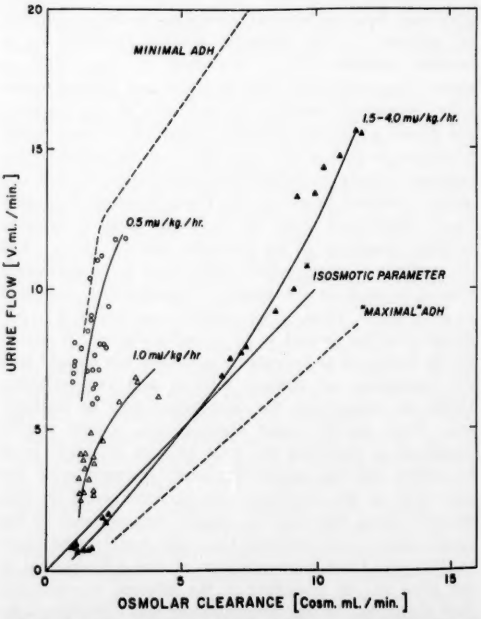



FIG. 2. THE RELATIONSHIP BETWEEN URINE FLOW AND SOLUTE EXCRETION DURING THE ADMINISTRATION OF GRADED DOSES OF VASOPRESSIN

[2] In one study, it was necessary to administer 4 mU per Kg. per hour of vasopressin to effect a marked fall in $C_{H_2O}$.

TABLE II

*Effect of vasopressin dosage on free water clearance (Dog T., 19 Kg.)*

| Time | Urine flow | $C_{osm}$ | $C_{H_2O}$ | $C_{Cr}$ | $P_{osm}$ |
|---|---|---|---|---|---|
| *min.* | *ml./min.* | | | | *μosm/Kg. $H_2O$* |
| 0 | 570 ml. $H_2O$ administered by stomach tube | | | | |
| 14 | Infuse 15 mg./min. creatinine in 5.0 ml./min. of 2.5% dextrose in $H_2O$ | | | | |
| 123–133 | 10.8 | 1.6 | 9.2 | 98 | 275 |
| −143 | 11.2 | 1.6 | 9.6 | 96 | 274 |
| −153 | 11.3 | 1.5 | 9.8 | 94 | 274 |
| 158 | Begin infusion of 1.5 mU/Kg./hr. of arginine vasopressin | | | | |
| | Decrease dextrose infusion to 0.5 ml./min. | | | | |
| 153–163 | 1.9 | 2.1 | −0.20 | 102 | 259 |
| −173 | 2.1 | 2.3 | −0.20 | 102 | 259 |
| −183 | 1.7 | 2.2 | −0.50 | 101 | 259 |
| 185 | Begin infusion of 5% mannitol at 4.6 ml./min.; dextrose at 4.5 ml./min. | | | | |
| 220–230 | 6.9 | 6.5 | 0.40 | 108 | 260 |
| −240 | 7.5 | 6.8 | 0.70 | 108 | 260 |
| −250 | 7.7 | 7.1 | 0.60 | 106 | 261 |
| 255 | Begin infusion of 6.5 mU/Kg./hr. of arginine vasopressin | | | | |
| 250–270 | 7.9 | 7.4 | −0.5 | 113 | 262 |
| −290 | 6.0 | 8.0 | −2.0 | 104 | 262 |
| −312 | 5.3 | 8.3 | −3.0 | 108 | 260 |
| −322 | 5.1 | 7.7 | −2.6 | 106 | 260 |

rates of vasopressin administration over the range of solute excretion examined in these studies.[3] The transition in urine concentration from hypertonic to hypotonic observed when 1.5 to 4.0 mU per Kg. per hour of vasopressin were administered is clearly due to the insufficiency of the vasopressin dosage in the face of an acute rise in solute excretion. The argument is further supported by the results of studies in which a reversal of the transition, *i.e.*, the reestablishment of hypertonicity, was observed when additional vasopressin was administered. The details of one such study are presented in Table II. A summary of the experiments is tabulated in Table III.

## DISCUSSION

It had been assumed for many years that the major action of vasopressin was to induce the elaboration of a hypertonic urine. Recently Smith (3) and Wesson and Anslow (4) suggested that rather than promote active reabsorption of water from either hypotonic or isotonic fluid, vasopressin acts to increase tubule membrane permeability to water in the distal segment, thereby permitting outward diffusion of water along an osmotic gradient. Their view of the sequence of events in urinary dilution and concentration is as follows: Dilution, the freeing of water from isotonic urine, is effected by abstraction of sodium and anion in an area of tubule *impermeable* to water. In the presence of antidiuretic hormone, the tubule membrane in this segment becomes freely permeable to water. Water diffuses out along the osmotic gradient developed by electrolyte abstraction until equilibrium is achieved. Elaboration of hypertonic urine is presumably accomplished by water abstraction from residual isosmotic urine in a more distal area. Whether this last process was influenced by ADH had not been determined. That vasopressin affects the permeability of the tubule membrane to water is in accord with the observations of Koefoed-Johnsen and Ussing (9) in frog skin. These investigators noted an increase in net water flux across isolated frog skin under the influence of neurohypophyseal extract and concluded on the basis of this and other data that neurohypophyseal hormones produce this effect by increasing pore size within the skin. Despite acceptance of this as the probable mode of action of vasopressin in the renal tubule as well as in frog skin, it is not possible to interpret all of the findings observed

TABLE III

*Effect of vasopressin dosage and solute excretion on clearance of free water* *

| Experiment no. | Submaximal ADH† | | Solute diuresis Submaximal ADH† | | Solute diuresis "Maximal" ADH‡ | |
|---|---|---|---|---|---|---|
| | $C_{osm}$ | $C_{H_2O}$ | $C_{osm}$ | $C_{H_2O}$ | $C_{osm}$ | $C_{H_2O}$ |
| 1 | 2.2 | −0.5 | 6.8 | 0.7 | 8.3 | −3.0 |
| 2 | 1.1 | −0.4 | 9.7 | 1.1 | 12.2 | −1.6 |
| 3 | 1.7 | −0.9 | 10.9 | 3.8 | 11.1 | −0.9 |
| 4 | 1.1 | −0.2 | 11.5 | 4.1 | 12.1 | −1.1 |

* Each datum represents a single period during the course of the study.

† Initial (submaximal) vasopressin dosage varied between 1.5 to 4.0 mU per Kg. per hour of purified vasopressin.

‡ "Maximal" vasopressin dosage varied from 6.5 mU per Kg. per hour of purified vasopressin to 600 mU per Kg. per hour of commercial vasopressin.

[3] In contrast to the results of these studies, the excretion of a hypotonic urine has been observed in some studies during the administration of presumably adequate amounts of commercial vasopressin (Pitressin®). In these both a rise in plasma osmolality and massive osmotic diuresis were induced by the administration of hypertonic mannitol or saline solutions (8).

in this and other studies (2, 10) strictly within the framework of Smith's and Wesson and Anslow's hypothesis. One would expect, were the membrane freely permeable to water during vasopressin administration, that superimposed osmotic diuresis would invariably result in some degree of urine hypertonicity. It is likely that: 1) permeability to water is never absent, and therefore that the rate of outward diffusion of water is dependent on intraluminal osmotic pressure even during water diuresis (11); and 2) the permeability of the tubule membrane in the distal segment varies in a graded fashion as a function of the dosage of vasopressin (12).

The first of these arguments, *i.e.*, the partial permeability to water even in the absence of ADH, has been discussed by Orloff and Walser (11). They observed a progressive rise in $C_{H_2O}$ during combined water and solute diuresis in patients with nephrogenic diabetes insipidus, and concluded that this was due in part to a progressive decline in the rate of diffusion of freed water out of the tubule as a consequence of the osmotic restraint of nonabsorbed solute in tubule urine. In other terms, the presence of solute in tubule urine decreases the activity of water and thereby its rate of outward diffusion.[4] The results of a number of other studies (13, 14) as well as those reported here in dogs during ADH suppression are consistent with this view. In the presence of large amounts of vasopressin, on the other hand, permeability may be sufficient to permit equilibration of water on both sides of the tubule membrane despite the opposing influence of intratubular solute. Under these circumstances the terminal concentrating process (see below) will effect the excretion of a hypertonic urine.

Intermediate dosages of vasopressin will produce effects dependent on the degree of membrane permeability achieved and the extent of the solute diuresis. The studies of Hollander, Williams, Fordham, and Welt (12) clearly indicate that vasopressin does affect membrane permeability in a graded fashion. The results observed in these studies may be similarly interpreted. Consequently, the observation that initial hypertonicity of urine is not maintained as solute excretion rises, unless greater amounts of hormone are administered, indicates that under these circumstances (submaximal ADH activity) membrane permeability is insufficient to permit complete osmotic equilibration. Presumably both flow and the osmotic gradient are such as to limit the back-diffusion of water, permitting delivery of a larger volume of hypotonic urine to the concentrating site. Thus, during submaximal ADH activity and osmotic diuresis, a part of the water "freed" diffuses out of the urine in both the segment in which free water is produced and in the terminal concentrating area. As vasopressin dosage increases, although the rate at which solute is reabsorbed to produce free water need not necessarily change, more water leaves the tubule by diffusion as a consequence of a further increase in membrane permeability. Urine will become hypertonic at differing levels of solute excretion depending upon vasopressin dosage. In essence it is suggested that solute excretion, insofar as it affects intraluminal osmotic pressure, and vasopressin have opposing effects, the former diminishing outward diffusion of "freed" water and the latter enhancing it. Which effect is greater will determine whether urine is hypotonic or isotonic when delivered to the segment in which water can be removed to produce hypertonicity. The presumption in these studies was that once pore size became "maximal," osmotic equilibrium was achieved at all rates of flow and solute excretion examined, and urine hypertonicity developed uniformly. This does not preclude the probability that at even higher rates of solute excretion back-diffusion of water may lag sufficiently to permit excretion of hypotonic urine despite "maximal" vasopressin activity. Whether the data obtained during massive osmotic diuresis in dogs in which plasma osmolality was elevated (8), as well as the data of Anslow and Wesson (10), may be explained on this basis as had been suggested by Mudge, Foulks, and Gilman (15) has not yet been determined.

The elaboration of hypertonic urine requires terminal abstraction of water in a tubule area relatively impermeable to solute. Though this process may occur to a limited degree in the absence of

[4] It is also recognized that the rise in $C_{H_2O}$ observed during solute diuresis could be accounted for on the basis of increased NaCl reabsorption. Though this possibility cannot be ruled out, and an increase in NaCl reabsorption may contribute to some of the changes observed, the osmotic effect of solute must be of significance in this regard (11).

hormones (16) the inability to excrete maximally concentrated urine under the conditions of the experiments reported by Berliner and Davidson (16) is consistent with the view that the terminal concentrating process may also be enhanced by ADH. This would presumably entail an increase in water permeability in this area as well as at the diluting site, thereby permitting increased outward diffusion of water. This is in accord with the hypothesis of Wirz (17) who suggested that terminal concentration is the result of equilibration of collecting duct urine with medullary interstitial fluid, the concentration of which is greatly in excess of plasma.

Development of urine hypotonicity during vasopressin administration and osmotic diuresis has been observed by de Wardener and del Greco (2) in patients with diabetes insipidus, and by Anslow and Wesson (10) in dogs. The first group of authors recognized that this was due to insufficiency of vasopressin dosage. They concluded, however, that contrary to current views urine leaving the proximal segment is always hypotonic. This was based on the inference that osmotic diuresis effects the delivery of greater amounts of proximal urine to the concentrating site and that vasopressin merely functions to promote terminal abstraction of water from such dilute pre-urine. It is apparent that their data as well as those reported above may be interpreted within the context of the thesis that proximal urine is isosmotic and that vasopressin promotes outward diffusion of water throughout the distal segment. Anslow and Wesson (10), on the other hand, ascribed their results to Pitressin® "insensitivity" of unknown origin. Though their studies are not completely analogous to those reported here, the phenomenon may have been the result of submaximal amounts of hormone in the presence of solute diuresis. The dosage of vasopressin used by these investigators was not as great as that required in the present studies to promote persistent urine hypertonicity nor were precautions taken to prevent deterioration of vasopressin (acidification to pH 5.0). Furthermore, the extent of the solute diuresis was in excess of that observed in these experiments.

It is not possible to extend the views of Shannon (18) and Lauson (19) on the physiological rate of secretion of hormone on the basis of these data other than to point out that one cannot assign an absolute value to the amount of endogenous or exogenous hormone required to promote an effect unless all conditions are clearly defined. Numerous factors, including solute excretion, glomerular filtration rate, the state of bodily hydration, and possibly adrenal activity, may affect urine osmolality directly or indirectly, and thereby may modify the character of the observed response to vasopressin. If one views vasopressin as an agent which merely alters membrane permeability in the diluting and concentrating areas of the kidney, then it is apparent that one cannot assess its relative activity under all circumstances merely by observing changes in urine flow and concentration.

## SUMMARY AND CONCLUSIONS

The administration of varying dosages of purified vasopressin during combined water and solute diuresis in dogs results in a progressive decline in $C_{H_2O}$ dependent on the dosage of hormone. The graded effect is such that at low rates of vasopressin administration a transition from hypertonic to hypotonic urine is noted as solute excretion rises. This transition may be reversed with maximally effective dosages of the hormone.

The data have been interpreted in the light of the thesis that vasopressin affects tubule membrane permeability in a graded fashion throughout the distal segment, both in the area where free water is present and in the terminal concentrating site. Osmotic equilibration of tubule fluid with renal interstitial tissue is essential for the elaboration of hypertonic urine. Since solute excretion and vasopressin exert opposing effects on the movement of water out of the tubule lumen into the interstitial space, net movement of water and the final urinary solute concentration are dependent on the relative contribution of each of these factors to the overall process.

## REFERENCES

1. Adolph, E. F., and Ericson, G. Pituitrin and diuresis in man. Amer. J. Physiol. 1927, **79**, 377.
2. de Wardener, H. E., and del Greco, F. The influence of solute excretion rate on the production of a hypotonic urine in man. Clin. Sci. 1955, **14**, 715.
3. Smith, H. W. The Kidney, Structure and Function in Health and Disease. New York, Oxford Univ. Press, 1951.

4. Wesson, L. G., Jr., and Anslow, W. P., Jr. Effect of osmotic and mercurial diuresis on simultaneous water diuresis. Amer. J. Physiol. 1952, **170**, 255.
5. Kennedy, T. J., Jr., Hilton, J. G., and Berliner, R. W. Comparison of inulin and creatinine clearance in the normal dog. Amer. J. Physiol. 1952, **171**, 164.
6. Walser, M., Davidson, D. G., and Orloff, J. The renal clearance of alkali-stable inulin. J. clin. Invest. 1955, **34**, 1520.
7. Bowman, R. L., Trantham, H. V., and Caulfield, P. A. An instrument and method for rapid, dependable determination of freezing-point depression. J. Lab. clin. Med. 1954, **43**, 310.
8. Orloff, J., Wagner, H. N., Jr., and Davidson, D. G. Unpublished observations.
9. Koefoed-Johnsen, V., and Ussing, H. H. The contributions of diffusion and flow to the passage of $D_2O$ through the living membrane. Acta physiol. scand. 1953, **28**, 60.
10. Anslow, W. P., Jr., and Wesson, L. G., Jr. Effect of sustained, graded urea diuresis on water and electrolyte excretion. Amer. J. Physiol. 1955, **180**, 605.
11. Orloff, J., and Walser, M. Water and solute excretion in Pitressin-resistant diabetes insipidus. Clin. Res. Proc. 1956, **4**, 136.
12. Hollander, W., Jr., Williams, T. F., Fordham, C. C., III, and Welt, L. G. A study of the quantitative relationship between antidiuretic hormone (vasopressin) and the renal tubular reabsorption of water. J. clin. Invest. 1957, **36**, 1059.
13. Rosenbaum, J. D., Nelson, W. P., III, Strauss, M. B., Davis, R. K., and Rossmeisl, E. C. Variation in the diuretic response to ingested water related to the renal excretion of solutes. J. clin. Invest. 1953, **32**, 394.
14. Kleeman, C. R., Epstein, F. H., and White, C. The effect of variations in solute excretion and glomerular filtration on water diuresis. J. clin. Invest. 1956, **35**, 749.
15. Mudge, G. H., Foulks, J., and Gilman, A. Effect of urea diuresis on renal excretion of electrolytes. Amer. J. Physiol. 1949, **158**, 218.
16. Berliner, R. W., and Davidson, D. G. Production of hypertonic urine in the absence of pituitary antidiuretic hormone. J. clin. Invest. 1957, **36**, 1416.
17. Wirz, H. The location of antidiuretic action in the mammalian kidney *in* The Neurohypophysis, H. Heller, Ed. New York, Academic Press Inc., 1957, p. 157.
18. Shannon, J. A. The control of the renal excretion of water. J. exp. Med. 1942, **76**, 371.
19. Lauson, H. D. The problem of estimating the rate of secretion of antidiuretic hormone in man. Amer. J. Med. 1951, **11**, 135.

# CONTRACTILITY OF ACTOMYOSIN BANDS PREPARED FROM NORMAL AND FAILING HUMAN HEARTS[1]

By K. KAKO and R. J. BING

*(From the Department of Medicine, Washington University School of Medicine, and the Washington University Medical Service, Veterans Administration Hospital, St. Louis, Mo.)*

(Submitted for publication July 25, 1957; accepted November 14, 1957)

Previous work from this laboratory has indicated that actomyosin bands prepared from heart muscle of dogs preserve undiminished contractility for one hour after the death of the animal (1). This suggested the possibility of investigating the contractile properties of actomyosin bands from human hearts obtained at autopsy. Such a study would make possible a comparison of actomyosin prepared from normal human heart muscle with that obtained from heart muscle of patients who had died of congestive heart failure. The method could also be used to obtain information on the effect of cardiac glycosides and other drugs on the contractile elements of normal and failing human heart muscle. To the present, only actomyosin preparations from hearts with artificially produced failure have been studied (2, 3). In these "models" obtained from dogs' hearts, the mechanism and nature of failure may vary from that encountered in man.

The present report deals with a comparison of the contractile properties of actomyosin prepared from normal and failing human hearts and with the effect of calcium chloride and digoxin and the action of nicotine on these preparations.

## MATERIAL AND METHODS

Actomyosin solution was prepared in the cold (4° C.) as follows: The heart was minced with scissors and 10 Gm. of the muscle was homogenized in a Waring blendor for three minutes with a fourteenfold volume of Weber-Edsall solution. The muscle was then extracted for 24 hours at 6° C. after addition of 20 mg. of the disodium salt of adenosine triphosphate (ATP). After extraction, the homogenate was mixed gently and 150 ml. of Weber-Edsall solution was added. The mixture was then centrifuged in the cold at 4,000 rpm for 15 minutes. The supernatant fluid was again centrifuged at 4,000 rpm for 10 minutes. Following this, the supernatant fluid was diluted with a fivefold volume of triple glass distilled water; the pH was adjusted to 7.0 by careful addition of 1/10 N acetic acid. Usually, 17 ml. of the acid was sufficient. The solution was then centrifuged at 1,500 rpm for 30 minutes. The supernatant was discarded and the precipitate was suspended in about 20 ml. of triple glass distilled water and centrifuged again for 15 minutes. The precipitate was dissolved in 2 M KCl to a final concentration of 0.5 M KCl. One ml. of this solution was used for the formation of actomyosin bands. The solution was pipetted on the surface of a glass slide, from which it was transferred into a solution contained in a Langmuir trough. This trough solution contained 0.05 M KCl and 0.001 M magnesium chloride, and was buffered to a pH of 7.5 with 1/100 M barbital buffer. The temperature in the trough was kept at 24° C.

The technique for the formation of bands has been previously described (4). However, since certain modifications were used, the method is briefly summarized at this point. The protein solution spread on the surface of the trough was compressed between two wooden sticks into bands, and then pushed gently into the contraction chamber containing 40 ml. of the trough solution (Figure 1). One end of the band was firmly fixed in position by a rigid connection with the bottom of the trough (Figure 1). The other end of the band was attached to the elongated arm of a Roller Smith torsion balance. The length of the band was approximately 2 cm., the width about 5 mm. Only afterloaded contractions were studied. The band was loaded by moving the weighing spring of the torsion balance by the desired number of mg. (between 5 to 25 mg.). ATP was now added to the contraction chamber to make a final concentration of 0.005 M. As the protein band contracted upon the addition of ATP, the arm of the torsion balance followed the contraction. This initiated an electronic Servo feedback mechanism which moved the trough in a direction opposite to that of the contraction. Thus, the arm of the torsion balance was always kept in equilibrium position and a counter force was produced which equalized in every instance the tension of the thread. The movements of the trough were recorded on paper with an inkwriter. With this method, one can record without appreciable friction, shortening of the band at a magnification of 23 diameters. Percentage of shortening of the bands was calculated by dividing the amount of contrac-

[1] Work supported by United States Public Health Service Grant No. H-2678, The Life Insurance Medical Research Fund; The American Heart Association; Burroughs Wellcome and Co., Inc.; and The Tobacco Industry Research Fund.

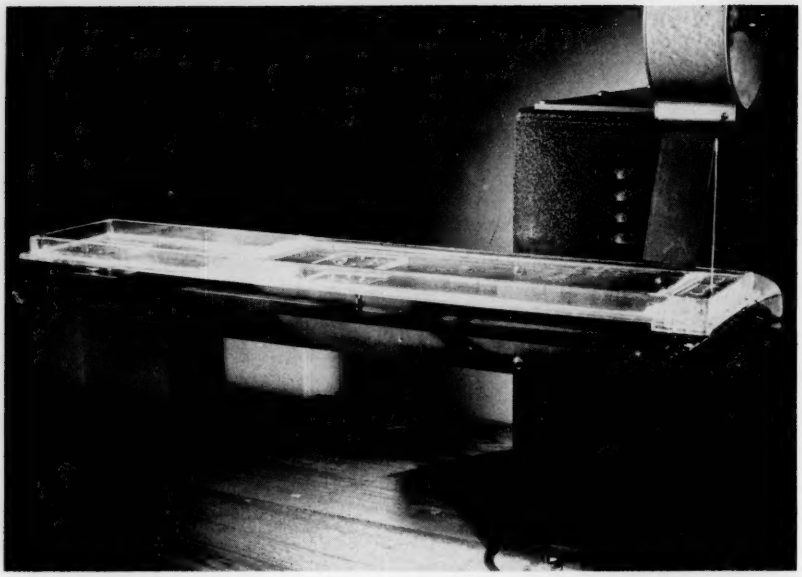


FIG. 1. THE LANGMUIR TROUGH IN WHICH THE ACTOMYOSIN SOLUTION IS COMPRESSED INTO BANDS

The contraction chamber is at the right. The torsion balance and its elongated arm are also shown. As the protein band contracts, the elongated arm of the torsion balance moves with the contraction; the trough moves in an opposite direction. Thus, the counter force produced equalizes the tension on the bands.

tion (mm.) by the initial length of the band 30 minutes after addition of ATP. All contractions were expressed per mg. of load. Results were subjected to statistical analysis. Regression lines were calculated for each series of experimental conditions and the significance of the regression coefficient was obtained with standard procedures (5). The group means and regression coefficients thus obtained were tested for significant differences from the control group (hearts obtained from patients without heart failure, without addition of digitalis, calcium chloride, or nicotine). To determine whether the interval between death of the patient and time of extraction of actomyosin alters the contraction of the bands, individual regression lines were obtained for each of these time intervals, and the regression lines were tested for significant differences between both the group means and the regression coefficient (5).

Material was obtained at autopsy from human heart muscle. Ten to twenty Gm. of muscle were excised, the pericardium was discarded, and the muscle was chopped into small pieces and placed in crushed ice. Homogenates were then made and preparation of actomyosin was carried out in the manner described above. The material was collected from 41 hearts at periods ranging from one to six hours after death of the patient. Twelve of the patients had died from congestive heart failure due to arteriosclerotic or hypertensive heart disease. The remainder died from malignancy, cirrhosis of the liver, leukemia, peptic ulcer, or cerebral vascular accident.

## RESULTS

Before comparative studies on contraction of actomyosin bands from human hearts could be carried out, it had to be ascertained that prolonged intervals between death of the patient and preparation of actomyosin did not affect the contractility of the protein. The experiments revealed no significant differences between the fresh preparation and that obtained from one to six hours after death. (Differences among the group mean $p > 0.05$.) This applies to actomyosin prepared from both normal and failing human hearts.

Figure 2 illustrates the findings obtained on actomyosin prepared from heart muscle of patients who had died without clinical evidence of heart failure. As the load on the protein band increased, the percentage shortening diminished. The relationship between load and percentage shortening was maintained after nicotine had been added to the contraction chamber (Figure 3).

However, the degree of shortening of the bands was less per mg. load than in the control series (Figure 3). The effect of digoxin with and without the addition of calcium chloride as well as of calcium chloride alone is illustrated in Figure 4. Neither calcium chloride nor digoxin appeared to influence the contractility of the protein band. However, calcium chloride in combination with digoxin increased the contractility of actomyosin prepared from normal human hearts. Most of the values lie on the positive side of the regression line and several are above the standard deviation of the normal. The slope of the regression line is

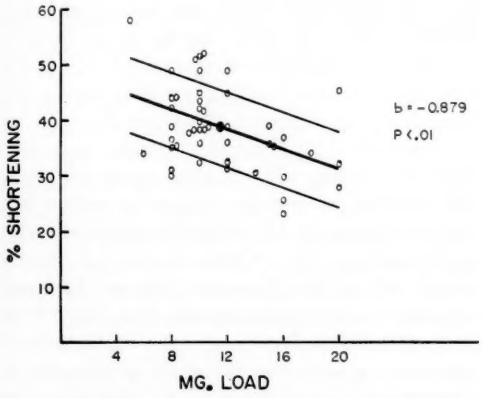



FIG. 2. THE RELATIONSHIP BETWEEN LOAD AND PERCENTAGE SHORTENING OF NONFAILING HUMAN HEARTS

It may be seen that the scatter of data is moderate and that there is a significant relationship between load and percentage shortening. The large black symbol represents the group mean, the heavy center line is the regression line, the two lines parallel to it are the standard deviations.

slightly less than that of the control values; the group mean, on the other hand, is higher than that of the control. This difference is statistically significant ($< 0.05$). Similar results were obtained by Korey and Edman, who demonstrated that the simultaneous addition of calcium chloride and ouabain increased the contractility of glycerinated skeletal muscle strips by 33 per cent (6, 7).

The results on 45 actomyosin bands prepared from hearts of patients who died in congestive heart failure are illustrated in Figure 5. Protein bands prepared from these hearts showed a significant reduction in percentage shortening.

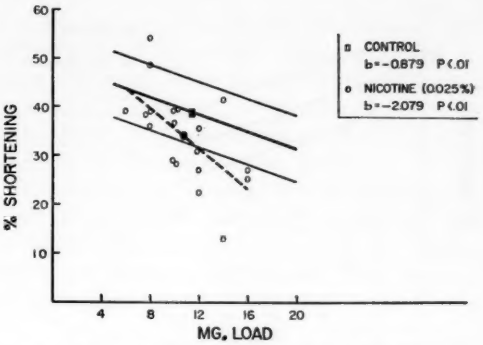



FIG. 3. THE EFFECT OF NICOTINE ON THE RELATIONSHIP BETWEEN LOAD AND PERCENTAGE SHORTENING

The broken line represents the regression line obtained from the data obtained with nicotine. The slope of this line is steeper than that of control and the group mean is lower. The regression line and standard deviations obtained from normal data are again represented.

Many of the data lie below the standard deviation of the normal controls. As in normal hearts, digoxin failed to increase the contractility of actomyosin prepared from failing human hearts (Figure 5). In contrast, the combined addition of digoxin plus calcium chloride resulted again in a significant improvement in contraction of the actomyosin bands (Figure 5). This is illustrated

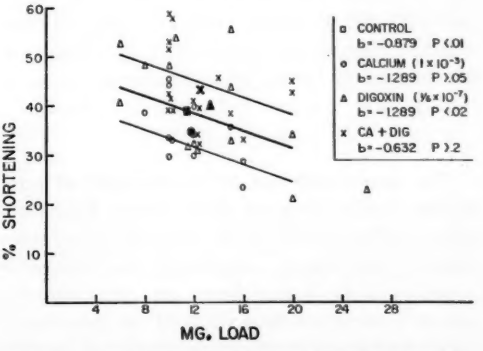



FIG. 4. THE EFFECT OF CALCIUM, DIGOXIN, AND CALCIUM AND DIGOXIN COMBINED ON ACTOMYOSIN BANDS FROM NORMAL HEARTS

The normal regression line and standard deviations are again represented. The group means of calcium and digoxin added separately (indicated by the heavy mark) are close to the group means of the normal. The group mean for calcium and digoxin combined is significantly higher than the normal group mean.

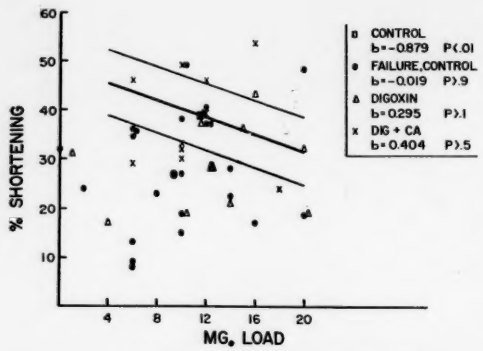



FIG. 5. THE CONTRACTILITY OF ACTOMYOSIN BANDS PREPARED FROM FAILING HUMAN HEARTS AND THE EFFECT OF DIGOXIN, AND OF CALCIUM AND DIGOXIN COMBINED

The regression line and standard deviations obtained from normal data are again represented. Many of the points obtained from failing hearts are below the standard deviation of the normal. Digoxin does not influence the percentage shortening (the group mean is still below the deviation of normal data). The combination of digoxin and calcium results in marked improvement of contractility as indicated by the fact that the group mean is now on the normal regression line.

by the fact that the group mean of this experimental series is 39 per cent for a load of 11.8 mg. This is identical with the group mean of the values obtained from normal hearts (Figure 1). Apparently, the glycoside combined with calcium chloride restores the contractility of actomyosin bands of failing human hearts to normal.

## DISCUSSION

The data reveal that the contractility of actomyosin bands prepared from human hearts remains undiminished for at least six hours after death of the patient. Apparently, the contractile proteins of the human heart are even more resistant to autolysis than are ATP or the respiratory enzymes of heart muscle which are inactive three hours after death (8, 9).

A reverse relationship exists between load and percentage shortening of actomyosin bands prepared from human heart muscle (Figure 2). This relationship is maintained after the addition of nicotine, although shortening of the bands is less than in the control series (Figure 3). The effect of nicotine is so slight that a direct effect of this drug on the contractile proteins of the heart cannot be postulated. This observation is in line with previously published data on the effect of nicotine on the isolated heart (10, 11).

Digoxin and calcium chloride, when added separately to the actomyosin solution or the contraction chamber, fail to influence the contractility of actomyosin bands prepared from either normal or failing human hearts. These observations are in agreement with those obtained by several authors. Korey, for example, demonstrated that digoxin does not change shortening of glycerinated skeletal muscle strips and Stutz, Feigelson, Emerson, and Bing have shown that lanatoside-C influences neither the force nor the speed of contracting glycerinated heart muscle (6, 12). No effect of digoxin on the length-resting tension relationship of glycerinated heart muscle could be demonstrated (12). Similar results were obtained by Edman (7); Benson found that digitalis does not reverse the changes in cardiac actomyosin observed in artificially produced congestive failure (3). Other workers, however, noticed that cardiac glycosides influence the speed and force of actomyosin threads (13, 14). It is likely that these discrepancies are the result of differences in technique and in the preparation of actomyosin.

The results reported in this paper illustrate that the combination of calcium and glycoside leads to an increase in contractility of actomyosin bands prepared from both failing and nonfailing human heart muscle. It has long been known that in the frog heart $CaCl_2$ is essential for the activity of cardiac glycosides. Loewi was of the opinion that the glycosides sensitize the myocardium to the action of calcium chloride (15). Potentiation of the effect of digitalis by calcium ion has been demonstrated in the frog heart, the Langendorff, and the heart-lung preparations (16–19). The mechanism of the synergistic action between $CaCl_2$ and the glycosides is not known. Wilbrandt is of the opinion that changes in extracellular $CaCl_2$ produce an alteration in the intracellular concentrations of the ion (20); the contractility of actomyosin would thus be increased by influencing the ATP-ase activity of this protein. Bozler has demonstrated that the glycerol extracted muscle

fiber takes up $CaCl_2$ from a dilute solution of this salt, but where and how $CaCl_2$ is bound to the muscle protein has not been ascertained (21). Ellis has shown, however, that the positive inotropic effect of $CaCl_2$ and digitalis is not dependent upon the functional integrity of oxidative metabolism of the cell (22).

Actomyosin bands prepared from failing human hearts demonstrate a marked diminution in contractility. It is not possible to state at this point whether this is the result of changes in contractile proteins of the failing heart. However, Benson had previously noted that in dogs with experimental heart failure there occurred a decrease in concentration of actomyosin, diminished viscosity per unit of actomyosin, and decreased viscosity response to the addition of ATP (3). He ascribed these changes to alterations in the organization of actomyosin, more specifically to the presence of myosin uncombined with actin. The dissociated state of actomyosin may be due to physical changes in heart muscle. Olson concluded that heart failure in dogs leads to marked changes in the physical-chemical properties of myosin consistent with a sizable increase in molecular weight (2). The results reported in this paper, although confirming the existence of changes in actomyosin of failing hearts, permit no conclusions on the nature of these alterations. It is likely, however, that the observed differences between the contractility of actomyosin prepared from normal and failing hearts are due to changes in the heart muscle prior to extraction. It is difficult to visualize, for example, how the failing heart muscle could be more susceptible to autolysis than muscle prepared from nonfailing hearts.

## SUMMARY

Actomyosin bands were prepared from normal and failing human hearts obtained at necropsy. ATP-induced afterloaded contractions were recorded without friction by means of a new null seeking device.

The time interval (two to six hours) between death of the patient and the preparation of actomyosin did not affect the shortening of the actomyosin bands.

Actomyosin bands prepared from normal hearts demonstrated a reverse relationship between load and percentage shortening. Nicotine caused a slight diminution in the contractility of the bands, while $CaCl_2$ and digoxin, when added separately, had no effect. When combined, $CaCl_2$ and digoxin increased the shortening of the bands.

The contractility of actomyosin bands prepared from failing human hearts was significantly diminished. Digoxin failed to correct this defect, but the combination of digoxin and $CaCl_2$ restored shortening of the bands to normal.

## REFERENCES

1. Dettli, L., and Bing, R. J. Contractility and extractability of heart actomyosin after death. Circulat. Res. 1956, **4**, 519.
2. Olson, R. E. Editorial: Molecular events in cardiac failure. Amer. J. Med. 1956, **20**, 159.
3. Benson, E. S. Composition and state of protein in heart muscle of normal dogs and dogs with experimental myocardial failure. Circulat. Res. 1955, **3**, 221.
4. Dettli, L., and Bing, R. J. Contractile properties of actomyosin threads and bands prepared from dogs' hearts. Amer. J. Physiol. 1956, **187**, 145.
5. Snedecor, G. W. Statistical Methods. Ames, The Collegiate Press, Inc., 1946.
6. Korey, S. Some factors influencing the contractility of a nonconducting fiber preparation. Biochim. biophys. Acta 1950, **4**, 58.
7. Edman, K. A. P. The action of cardiac glycosides on the ATP-induced contraction of glycerinated muscle fibers. Acta physiol. scand. 1953–54, **30**, 69.
8. Bate-Smith, E. C., and Bendall, J. R. Changes in muscle after death. Brit. med. Bull. 1956, **12**, 230.
9. Beuren, A., Choudhury, J. D., and Bing, R. J. Studies on the persistence of metabolic functions on the heart after death. In preparation.
10. Mansfeld, G., and Hecht, K. Untersuchungen ueber die Wirkung des Tabakrauchens auf das Herz—Lungenpraeparat von Hunden. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1933, **172**, 362.
11. Strauss, L. H. Nicotinwirkungen und- schaedigungen. Ergebn. inn. Med. Kinderheilk. 1937, **52**, 375.
12. Stutz, H., Feigelson, E., Emerson, J., and Bing, R. J. The effect of digitalis (Cedilanid) on the mechanical and electrical activity of extracted and non-extracted heart muscle preparations. Circulat. Res. 1954, **2**, 555.
13. Robb, J. S., and Malloy, S. Effect of ouabain on the actomyosin threads. J. Pharmacol. exp. Ther. 1953, **108**, 251.
14. Bowen, W. J. Effect of digoxin upon rate of shortening of myosin B threads. Fed. Proc. 1952, 11, 16.

15. Loewi, O. Ueber den Zusammenhang zwischen Digitalis- und Kalziumwirkung. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1918, **82**, 131.
16. Nyiri, W., and du Bois, L. Experimental studies on heart tonics. III. The relationships of calcium ions, hydrogen ions and digitalis. J. Pharmacol. exp. Ther. 1930, **39**, 111.
17. Wiechmann, E. Ueber die Beseitigung von Giftwirkungen am Herzen durch Calcium und andere zweiwertige Kationen. Pflüg. Arch. ges. Physiol. 1922, **195**, 588.
18. Baker, W. W., and Baker, J. M. Effects of epinephrine and calcium on the electrogram of the ouabainized frog heart. Circulat. Res. 1955, **3**, 274.
19. Baker, J. B. E. The influence of calcium and potassium ions on the toxicity of ouabain. Brit. J. Pharmacol. 1947, **2**, 259.
20. Wilbrandt, W. Zum Wirkungsmechanismus der Herzglykoside. Schweiz. med. Wschr. 1955, **85**, 315.
21. Bozler, E. Binding of calcium and magnesium by the contractile elements. J. gen. Physiol. 1954–55, **38**, 735.
22. Ellis, S. The importance of metabolic pathways for the positive inotropic actions of cardiac glycosides and calcium ions. J. Pharmacol. exp. Ther. 1953, **109**, 233.

# EFFECT OF AORTIC INSUFFICIENCY ON ARTERIAL BLOOD PRESSURE, CORONARY BLOOD FLOW AND CARDIAC OXYGEN CONSUMPTION [1]

By RENÉ WÉGRIA, GERHARD MUELHEIMS,[2] JAMES R. GOLUB,[3] ROBERT JREISSATY, AND JIRO NAKANO[4]

*(From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Presbyterian Hospital, New York, N. Y.)*

(Submitted for publication September 19, 1957; accepted November 21, 1957)

In patients with aortic insufficiency and without disease of the coronary ostia or arteries, the occurrence of chest pain having some or many of the characteristics of cardiac pain due to myocardial ischemia has been ascribed classically to a decrease in the coronary blood flow (1, 2), the aortic insufficiency presumably decreasing the coronary blood flow by lowering the mean aortic diastolic pressure. However, the data available on the effect of aortic insufficiency on the coronary blood flow are contradictory. From acute experiments on the anesthestized dog, Green (3), as well as Green and Gregg (4), concluded that when the aortic insufficiency is marked enough to lower the aortic diastolic pressure, the increase in coronary blood flow occurring during systole does not compensate for the decrease in flow during diastole and the mean coronary flow decreases. On the other hand, Foltz, Wendel, and West (5), from measurements made on anesthetized dogs in which one or two aortic cusps had been torn three or more days previously, concluded that the coronary blood flow was increased over levels usually observed in normal dogs while oxygen consumption was greatly increased over normal levels. Because of the discrepancy in the opinions about the effect of aortic insufficiency on the coronary blood flow, it was thought advisable to reinvestigate this problem.

## METHODS

Eight dogs weighing between 17 and 40 kilograms were anesthetized by the intravenous administration of 10 ml. per kilogram of weight of a 1 per cent chloralose solution. In the first four dogs the trachea, both common carotid arteries and both external jugular veins were exposed through a midline incision and a Y-shaped glass tube inserted into the trachea. Under artificial respiration, the left hemithorax was opened, the pericardium incised and the heart suspended in a pericardial cradle. The method used to measure and continuously record the output of the left ventricle was a modification of that previously described in this laboratory (6). The proximal ends of the left subclavian artery and brachiocephalic trunk were cannulated. Then the aorta was clamped distally to the subclavian artery so that the blood ejected by the left ventricle was directed into an electromagnetic rotameter (7) and returned to the circulation via both carotid arteries cannulated distally as well as both femoral arteries cannulated proximally and distally. Since the coronary sinus drains blood essentially only from the left coronary artery (8, 9) and since, in a given animal, the coronary sinus blood flow represents a constant portion of the left coronary artery flow (8), measurement of the coronary sinus blood flow was used as a means of studying the left coronary artery blood flow. For that purpose, a cannula was introduced via the left external jugular vein into the coronary sinus and tied in place by a string passed around the coronary sinus. All the blood emerging from the coronary sinus through this cannula was directed into a second rotameter by means of which the coronary sinus blood flow was measured and continuously recorded. The coronary sinus blood was returned to the dog through a cannula introduced via the right external jugular vein down to the junction of the right auricle and the superior vena cava. Mean arterial blood pressure was recorded from the root of the aorta with a dampened Gregg manometer. Left ventricular output, mean aortic blood pressure and coronary sinus blood flow were continuously recorded on photographic paper. The heart rate was obtained by direct timing, from the blood pressure tracing or from an electrocardiographic tracing. In the other four dogs the output of the left ventricle was not measured and the coronary sinus flow was measured as in the first four dogs. The mean femoral arterial blood pressure was recorded with a dampened Gregg manometer and the aortic pressure curve was recorded from the root of the aorta with another optical manometer. The instrument used to produce acute aortic insufficiency was the um-

[1] This work was made possible by grants-in-aid from the New York Heart Association, the Sidney A. Legendre Gift and the Charles A. Frueauff Gift.

[2] Postdoctorate Fellow of the United States Public Health Service.

[3] Fellow of the Dazian Foundation for Medical Research.

[4] Research Fellow of the Hudson County (N. J.) Heart Association.

TABLE I

*Effect of aortic insufficiency on heart rate, arterial blood pressure, cardiac output, coronary blood flow, coronary blood arteriovenous oxygen difference and cardiac oxygen consumption*

| Exp. and dog no. | | Duration of insufficiency | Heart rate beat | Arterial blood pressure | | | Cardiac output | Coronary flow | Coronary A-V $O_2$ difference | Oxygen consumption |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | Syst. | Diast. | | | | |
| | | *sec.* | *min.* | *mm. Hg* | | | *ml./min.* | *ml./min.* | *vol. %* | *ml./min.* |
| | | | | Aortic | | | | | | |
| | C.* | | 136 | 150 | | | 1,220 | 59 | 15.5 | 9.2 |
| 1. | A.I.† | 235 | 160 | 135 | | | 830 | 80 | 15.4 | 12.4 |
| | R.‡ | | 152 | 140 | | | 1,000 | 57 | 15.9 | 9.1 |
| | C. | | 160 | 130 | | | 1,100 | 94 | 12.7 | 11.8 |
| 2. | A.I. | 316 | 160 | 110 | | | 960 | 113 | 13.4 | 15.1 |
| | R. | | 160 | 128 | | | 1,060 | 87 | 12.5 | 10.9 |
| | C. | | 167 | 97 | | | 810 | 49 | 15.8 | 7.7 |
| 3. | A.I. | 340 | 167 | 76 | | | 600 | 55 | 15.6 | 8.6 |
| | R. | | 176 | 94 | | | 675 | 50 | 15.5 | 7.7 |
| | C. | | 166 | 119 | | | 940 | 65 | 12.2 | 7.9 |
| 4. | A.I. | 214 | 170 | 105 | | | 940 | 78 | 12.7 | 9.9 |
| | R. | | 170 | 120 | | | 1,050 | 74 | 12.8 | 9.5 |
| | | | | Femoral | | | | | | |
| | C. | | 160 | 120 | | | | 38 | 17.3 | 6.6 |
| 5. | A.I. | 304 | 162 | 98 | | | | 45 | 17.4 | 7.8 |
| | R. | | 162 | 110 | | | | 40 | 17.9 | 7.2 |
| | | | | Femoral | Aortic | | | | | |
| | $C_1$§ | | 144 | 132 | 157 | 116 | | 68 | 16.2 | 11.0 |
| A | $A.I._1$‖ | 208 | 150 | 125 | 150 | 98 | | 76 | 16.1 | 12.0 |
| | $R_1$ | | 146 | 132 | 148 | 114 | | 68 | 16.2 | 11.0 |
| | $C_2$¶ | | 148 | 128 | 154 | 115 | | 67 | | |
| 6. | $A.I._2$ | 80 | 148 | 122 | 144 | 88 | | 79 | | |
| B | $A.I._3$ | 155 | 150 | 117 | 145 | 80 | | 80 | | |
| | $A.I._4$ | 210 | 150 | 118 | 148 | 76 | | 85 | | |
| | $A.I._5$ | 277 | 150 | 108 | 141 | 63 | | 87 | | |
| | $R_2$ | | 150 | 128 | 156 | 115 | | 67 | | |
| | $C_1$ | | 183 | 128 | 140 | 105 | | 61 | 14.9 | 9.1 |
| A | $A.I._1$ | 244 | 182 | 105 | 140 | 48 | | 70 | 16.4 | 11.5 |
| | $R_1$ | | 185 | 128 | 140 | 105 | | 61 | 14.9 | 9.1 |
| 7. | $C_2$ | | 186 | 122 | 140 | 105 | | 57 | | |
| | $A.I._2$ | 85 | 188 | 104 | 142 | 52 | | 72 | | |
| B | $A.I._3$ | 243 | 188 | 104 | 145 | 64 | | 72 | | |
| | $A.I._4$ | 348 | 188 | 104 | 142 | 64 | | 70 | | |
| | $R_2$ | | 188 | 122 | 140 | 112 | | 61 | | |
| | $C_1$ | | 112 | 160 | 183 | 132 | | 65 | 21.1 | 13.7 |
| A | $A.I._1$ | 126 | 140 | 128 | 154 | 55 | | 83 | 21.5 | 17.8 |
| | $R_1$ | | 112 | 160 | 178 | 134 | | 74 | 21.3 | 15.8 |
| | $C_2$ | | 112 | 158 | 182 | 125 | | 62 | | |
| 8. | $A.I._2$ | 30 | 120 | 139 | 172 | 86 | | 81 | | |
| | $A.I._3$ | 83 | 122 | 156 | 172 | 95 | | 72 | | |
| B | $A.I._4$ | 130 | 132 | 138 | 176 | 96 | | 77 | | |
| | $A.I._5$ | 204 | 138 | 139 | 185 | 70 | | 86 | | |
| | $A.I._6$ | 251 | 138 | 130 | 164 | 42 | | 89 | | |
| | $A.I._7$ | 288 | 140 | 126 | 165 | 38 | | 91 | | |
| | $R_2$ | | 138 | 160 | 180 | 120 | | 68 | | |

* C., control.
† A.I., aortic insufficiency.
‡ R., recovery.
§ $C_1$, control for part A.
‖ $A.I._{1,2,3}$, *etc.*, aortic insufficiency of severity increasing or decreasing stepwise.
¶ $C_2$, control for part B.

brella type valve spreader previously described by Wiggers and Maltby (10). While blood pressure, coronary sinus blood flow and in some experiments left ventricular output were being continuously recorded, the valve spreader was slipped into the aortic ring through the left ventricular wall. The introduction of the instrument into the aortic ring was without effect on blood pressure, left ventricular output and coronary sinus flow. Proper positioning of the instrument was judged by palpation and the correctness of the position of the instrument was checked by direct visualization at the end of each experiment.

In all eight experiments, after a control period an aortic insufficiency severe enough to lower definitely the mean and/or diastolic arterial blood pressure was induced and maintained from 126 to 340 seconds, after which a period of recovery was observed. Arterial and coronary sinus blood samples were drawn simultaneously at the end of the control period, the period of aortic insufficiency and during the recovery period. The oxygen content of the arterial and venous bloods was determined in duplicate by the method of Van Slyke and Neill (11). The oxygen consumption of that portion of the myocardium drained by the coronary sinus was calculated according to the following formula:

Oxygen consumption (expressed in ml. per minute) equals coronary sinus blood flow (expressed in ml. per minute) times coronary oxygen arteriovenous difference (expressed in ml. per cent) times $10^{-2}$.

In three of the eight experiments, after the completion of that part of the experiment in which the effect of aortic insufficiency on oxygen consumption of the myocardium, arterial blood pressure and coronary blood flow was studied, an attempt was made to induce aortic insufficiency, then to increase (or decrease) the severity of the aortic insufficiency stepwise in order to establish the effect of aortic insufficiency of progressively increasing (or decreasing) severity on arterial blood pressure and coronary blood flow.

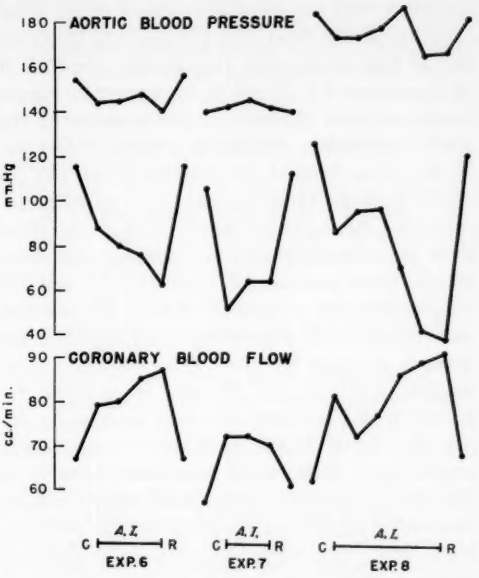



FIG. 1. PLOT OF PART B OF EXPERIMENTS 6, 7 AND 8, ILLUSTRATING THE EFFECT OF ACUTE AORTIC INSUFFICIENCY OF SEVERITY INCREASING (OR DECREASING) STEPWISE

Upper tracings, systolic and diastolic aortic blood pressure; scale in mm. of mercury. Lower tracing, coronary sinus blood flow; scale in ml. per minute.

C, control; A.I., aortic insufficiency of severity increasing (or decreasing) stepwise; R, recovery.

## RESULTS AND DISCUSSION

The results observed are summarized in Table I and Figure 1. The effect of aortic insufficiency on coronary blood flow and cardiac oxygen consumption was studied in eight experiments on eight dogs. In all eight experiments, aortic insufficiency lowered the mean arterial blood pressure, the decrease ranging from 7 to 32 mm. of mercury, and despite the sudden drop in mean arterial pressure the coronary flow rose in all eight experiments, the increase amounting from 12 to 36 per cent. The oxygen consumption rose in all eight experiments and this rise amounted from 9 to 35 per cent. In three experiments, this increase in oxygen consumption was due to the increase in coronary flow alone, the coronary blood oxygen arteriovenous difference remaining unchanged. In the other five experiments, most of the increase in oxygen consumption was contributed by the increase in coronary flow, although the coronary blood oxygen arteriovenous difference increased somewhat. The output of the left ventricle, *i.e.*, the effective cardiac output, was decreased in three of the four experiments in which it was measured and unchanged in one experiment. The heart rate was not at all or only slightly affected by the aortic insufficiency, except in two experiments in which it rose significantly. These latter two experiments were otherwise similar to the rest of the experiments. In Part A of Experiments 6, 7 and 8, in which the aortic pressure curve as well as the mean arterial femoral pressure was recorded, the mean arterial blood pressure was observed to fall from 132, 128 and 160 mm. of mercury to 125, 105 and 128 mm. of mercury, re-

spectively, and the diastolic pressure in the same experiments fell from 116, 105 and 132 to 98, 48 and 55 mm. of mercury, respectively. In Part B of Experiments 6, 7 and 8, illustrated in Figure 1, the stepwise *increase* in the severity of the aortic insufficiency resulted in a stepwise *decrease* in the mean arterial blood pressure as well as aortic diastolic pressure, and in a stepwise *increase* in the coronary flow. Part B of these three experiments shows that although the mean arterial blood pressure fell from 128, 122 and 158 mm. of mercury to minima of 108, 104 and 126 mm. of mercury, respectively, and although the diastolic pressure fell from 115, 105 and 125 to minima of 63, 52 and 38 mm. of mercury, respectively, the coronary flow rose stepwise to values 28, 15 and 47 per cent above control level, respectively. From these data there seems to be little doubt that acute aortic insufficiency increases the cardiac oxygen consumption and the coronary blood flow, even when the aortic insufficiency is severe enough to produce a marked decrease in mean arterial blood pressure and aortic diastolic pressure. As a matter of fact, within the limits of the present experiments, the more severe the aortic insufficiency was and the lower the mean arterial and aortic diastolic pressure fell, the more the coronary blood flow increased, as demonstrated by the second part of Experiments 6, 7 and 8.

These results are in disagreement with those of Green (3) and Green and Gregg (4), who, in acute experiments on anesthetized dogs, found that aortic insufficiency always decreased the coronary blood flow. Because partial occlusion of the aorta during aortic insufficiency could raise the coronary blood flow to or above control level by raising the mean aortic pressure, these authors postulated the possibility that, if aortic insufficiency induced sufficient peripheral vasoconstriction to restore the mean arterial pressure to its control level, aortic insufficiency could raise the coronary blood flow. However, they never actually observed such an occurrence, and in their experiments aortic insufficiency alone always lowered the coronary flow. It must be pointed out that the control arterial blood pressure of their animals was quite low and a decrease in the head of pressure at those very low levels of aortic pressure may have more of an effect on the coronary flow than the same decrease in the head of pressure at higher levels of aortic pressure. However, this would hardly seem to be the only factor responsible for the discrepancy between Green and Gregg's observations and the present study. Indeed, it should be kept in mind that the condition of dogs with as low a control arterial blood pressure as reported in the observations published by Green and Gregg is such that these dogs most probably respond to the induction of aortic insufficiency differently from dogs with a higher and more normal control arterial blood pressure as well as cardiovascular system in general. On the other hand, Foltz, Wendel, and West (5) observed that during aortic insufficiency "coronary blood flow was increased over levels usually observed in normal dogs, while oxygen consumption was greatly increased over normal values." Unfortunately, no control values for the blood pressure were given by these authors and it was therefore difficult to estimate the severity of the aortic insufficiency. Furthermore, the effect of the aortic insufficiency was appraised by comparison of the values of coronary blood flow found in a group of normal dogs with the values observed in the group of dogs with aortic insufficiency. Hence, no strict control values for coronary blood flow were available. The observations made on patients with aortic insufficiency are sparse and variable in their conclusions. Bing and his coworkers (12) reported coronary blood flow and cardiac oxygen consumption to be definitely increased in one case of aortic insufficiency as compared to a group of normal persons. Regan, Talmers, Christensen, Wada, and Hellems (13) reported that in three patients with severe aortic insufficiency the coronary flow was 91, 100 and 107 ml. per 100 grams of left ventricle per minute, and the oxygen consumption averaged 12.7 ml. per 100 grams of left ventricle per minute. In two patients with aortic insufficiency and angina pectoris the coronary blood flow was 66 and 68 ml., and the oxygen consumption 8 ml. per 100 grams of left ventricle per minute. These data were compared with data obtained on a group of 8 normal persons in whom the coronary blood flow was 103 plus or minus 20 ml. per 100 grams of left ventricle per minute, and the oxygen consumption was 10.5 plus or minus 2.6 ml. per 100 grams of left ventricle per minute. Such data are difficult to assess and hazardous to interpret

because of the small number of observations, the lack of adequate control data and the impossibility of ever ruling out the presence of complicating factors such as diseases of the coronary ostia and/or arteries.

In conclusion, it would seem that an acute aortic insufficiency severe enough to lower markedly the mean arterial blood pressure, as well as the aortic diastolic pressure, resulted in an increase in cardiac oxygen consumption and coronary blood flow. This increase in coronary flow must be due to a decrease in the resistance of the coronary bed, since the coronary flow rose despite a marked drop of the aortic pressure. This decrease in the resistance of the coronary bed was probably induced by the increase in the work of the left ventricle (14). Whether the increase in coronary flow was sufficient to meet the demands made upon the left ventricle by the aortic insufficiency, and thereby prevent myocardial ischemia, cannot be determined from these experiments.

## SUMMARY AND CONCLUSIONS

In the anesthetized dog acute aortic insufficiency, sufficient to lower markedly the mean arterial blood pressure and the aortic diastolic pressure, resulted regularly in an increase in coronary sinus blood flow and myocardial oxygen consumption.

## REFERENCES

1. Blumgart, H. L. Diseases of the coronary arteries. Angina pectoris *in* A Textbook of Medicine, R. L. Cecil and R. F. Loeb, Eds. Philadelphia, W. B. Saunders Co., 1955, p. 1321.
2. Harrison, T. R. Principles of Internal Medicine, 2nd ed. New York, Blakiston Co., 1954, p. 32.
3. Green, H. D. The coronary blood flow in aortic stenosis, in aortic insufficiency and in arteriovenous fistula. Amer. J. Physiol. 1936, **115**, 94.
4. Green, H. D., and Gregg, D. E. Changes in the coronary circulation following increased aortic pressure, augmented cardiac output, ischemia and valve lesions. Amer. J. Physiol. 1940, **130**, 126.
5. Foltz, E. L., Wendel, H., and West, J. W. Effects of aortic insufficiency on coronary blood flow and cardiac oxygen consumption (abstract). Fed. Proc. 1953, **12**, 44.
6. Wégria, R., Frank, C. W., Misrahy, G. A., Sioussat, R. S., McCormack, G. H., Jr., and Sommer, L. S. A new technic for the continuous recording of the cardiac output. Proc. Soc. exp. Biol. (N. Y.) 1950, **74**, 551.
7. Shipley, R. E., and Wilson, C. An improved recording rotameter. Proc. Soc. exp. Biol. (N. Y.) 1951, **78**, 724.
8. Gregg, D. E., and Shipley, R. E. Studies of the venous drainage of the heart. Amer. J. Physiol. 1947, **151**, 13.
9. Wégria, R., Frank, C. W., Wang, H. H., Blumenthal, M. R., Kornfeld, P., and Seely, R. D. Effect of elevation of right ventricular pressure on coronary circulation. Fed. Proc. 1954, **13**, 162.
10. Wiggers, C. J., and Maltby, A. B. Further observations on experimental aortic insufficiency. IV. Hemodynamic factors determining the characteristic changes in aortic and ventricular pressure pulses. Amer. J. Physiol. 1931, **97**, 689.
11. Van Slyke, D. D., and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. J. biol. Chem. 1924, **61**, 523.
12. Bing, R. J., Hammond, M. M., Handelsman, J. C., Powers, S. R., Spencer, F. C., Eckenhoff, J. E., Goodale, W. T., Hafkenschiel, J. H., and Kety, S. S. Measurement of coronary blood flow, oxygen consumption and efficiency of the left ventricle in man. Amer. Heart J. 1949, **38**, 1.
13. Regan, T. J., Talmers, F. N., Christensen, R. C., Wada, T., and Hellems, H. K. Coronary blood flow and myocardial metabolism in aortic insufficiency. Circulation 1956, **14**, 987.
14. Anrep, G. V., and Segall, H. N. The regulation of coronary circulation. Heart 1926, **13**, 239.

# THE CIRCULATORY AND METABOLIC EFFECTS IN MAN OF HISTAMINE, MECHOLYL®, TETRAETHYLAMMONIUM AND ATROPINE IN THE PRESENCE OF CIRCULATING EPINEPHRINE AND NOR-EPINEPHRINE [1]

By JOSEPH A. WILBER [2] and ALBERT A. BRUST [3]

*(From the Department of Internal Medicine, Emory University School of Medicine, and the Grady Memorial Hospital, Atlanta, Ga.)*

(Submitted for publication July 8, 1957; accepted November 14, 1957 )

In pheochromocytoma pharmacologic agents which provoke paroxysms of hypertension [*i.e.*, histamine (1), methacholine chloride (2), and tetraethylammonium chloride (3)] have come to be widely employed as clinical diagnostic tools. Although the usual effects of these drugs, namely fall in blood pressure, tachycardia and vasodilatation, are well recognized, the mechanism by which they induce hypertension in the pheochromocytoma patient remains unclarified.

This study was designed to evaluate the circulatory and metabolic responses induced by histamine, methacholine chloride (hereafter referred to as Mecholyl®) and tetraethylammonium chloride (TEAC) in the presence of measured amounts of circulating epinephrine and nor-epinephrine.

Atropine has been included as a test drug in an effort to determine the role which tachycardia may play in the observed responses, and also because Littman and co-workers (4, 5) have reported augmentation of nor-epinephrine's pressor effects by pretreatment with atropine.

## MATERIAL AND METHODS

*Blood pressure studies.* Fifty-four convalescent hospital patients free of cardiovascular and renal disease were studied during a series of 82 infusion experiments. Twenty patients had paired infusions including both epinephrine and nor-epinephrine.

The provocative test drugs employed and the dose and volume in which they were administered were:

1. Tetraethylammonium chloride,[4] 400 mg. (4 ml.) rapidly intravenously.
2. Histamine base, 0.025 mg. as histamine phosphate (0.35 ml.) of a 1:5,000 dilution rapidly intravenously.
3. Mecholyl®,[5] 10 mg. (1 ml.) subcutaneously.
4. Atropine sulphate,[6] 1.2 mg. (3 ml.) rapidly intravenously.

Small amounts of normal saline were infused during the initial and terminal control periods and the change to pressor amine was made by way of a three-way stopcock.

All tests were done in the supine position and in the fasting state at least 30 minutes after the intravenous needles and other apparatus had been arranged. Blood pressure was measured by the auscultatory method and pulse was counted every four minutes.

After a 10 minute control period, a control dose of the provocative test drug was given. Blood pressure was then followed at one-half minute intervals for five minutes and at one minute intervals thereafter. Thirty minutes after the control test, the infusion of epinephrine [7] or of nor-epinephrine [7] was begun. Fifteen minutes were allowed for stabilization of blood pressure and pulse at new levels; then the test drug was given again and the same observations repeated. Fifteen minutes later the infusion was switched to saline and blood pressure and pulse followed until control levels or a steady state were attained.

[1] This work was supported in part by grants from the United States Public Health Service, the Georgia Heart Association and the American Heart Association.

[2] Present address: Radcliffe Infirmary, Oxford, England. During the period of this study, Research Fellow of the American College of Physicians and later of the United States Public Health Service.

[3] Present address: The Kettering Laboratory, College of Medicine, University of Cincinnati, Cincinnati, Ohio. All requests for reprints should be directed to Dr. Brust at this address.

[4] Etamon® chloride, kindly supplied by Dr. E. C. Vonder Heide, Parke Davis and Co.

[5] Mecholyl®, kindly supplied by Merck and Co., Inc.

[6] Atropine sulfate, kindly supplied by Eli Lilly and Co.

[7] Pure synthetic *l*-epinephrine (Suprarenin®) and *l*-norepinephrine (Levophed®) kindly supplied by Dr. M. L. Tainter, Winthrop-Stearns, Inc., were diluted appropriately with saline in sterile volumetric pipettes and were infused at a constant rate of 0.9 ml. per minute by means of a Bowman constant infusion pump. Doses were so calculated that 0.085 μg. per Kg. per minute of epinephrine base or nor-epinephrine base was delivered. In a few early experiments larger doses were employed.

Atropine control tests were done on separate days because of the longer duration of drug action.

Blood samples for glucose and lactic acid were drawn at the following intervals: two control specimens, 15 minutes after the control dose of the provocative test drug, 15 minutes after the infusion of epinephrine or nor-epinephrine was begun, and 15 minutes after the test drug had been given during the infusion. Blood glucose was determined by the method of Nelson (6) and blood lactic acid by a modification of the method of Barker-Summerson (7).

*Cardiac output studies.* In 33 patients, only nor-epinephrine was infused and the responses to either TEAC or atropine observed. The cardiac output was measured during the infusion of nor-epinephrine and again shortly after the addition of either TEAC or atropine, using Hamilton's dye dilution technique with Evans blue dye. Serial blood samples were collected manually in heparinized tubes at one second intervals from an inlying brachial artery needle. The serum from the centrifuged specimens was transferred directly to microcuvettes and read in a Beckman spectrophotometer at 620 mμ. The results were plotted on semi-logarithmic paper and the cardiac output calculated using the formula of Hamilton and co-workers (8). All curves wherein the point of recirculation could not be definitely found or where

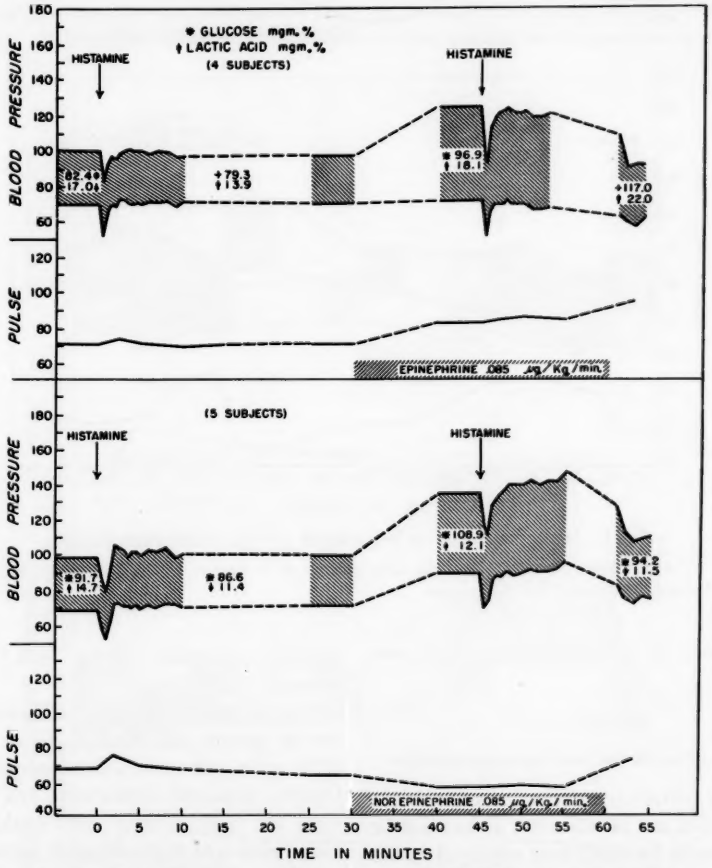



FIG. 1. MEAN RESPONSES TO HISTAMINE BASE (0.025 MG. INTRAVENOUSLY)

Mean blood pressure and pulse responses along with mean glucose and lactic acid values are indicated during control periods (far left) and during epinephrine infusion (upper right) and nor-epinephrine infusion (lower right). The unshaded areas and dotted lines indicate waiting periods during which blood pressure was allowed to stabilize, and do not represent actual values. Note that histamine remains depressor throughout.

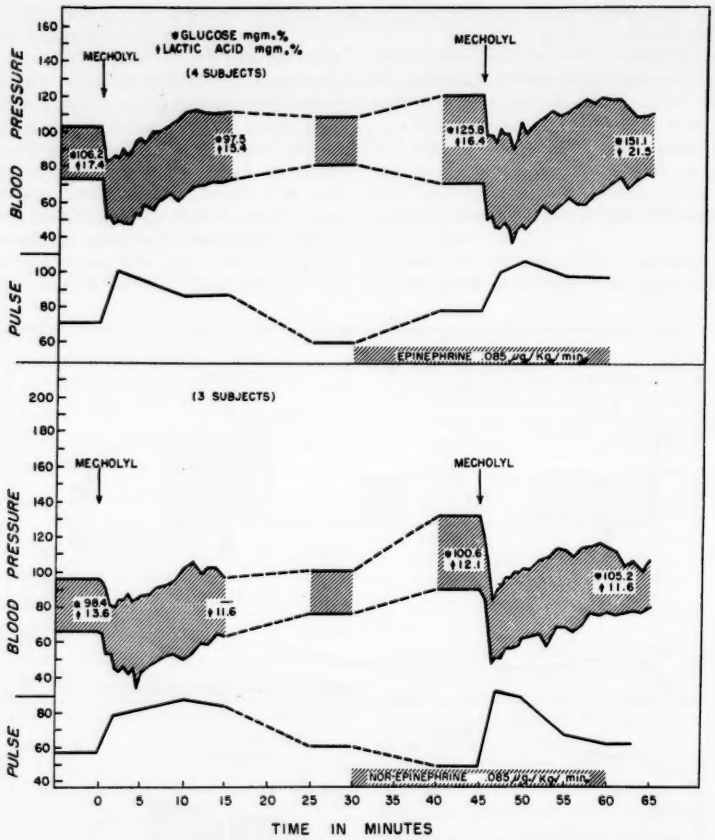



FIG. 2. MEAN RESPONSES TO MECHOLYL® (10 MG. SUBCUTANEOUSLY)

The data have been charted in the same manner as in Figure 1. As with histamine, Mecholyl® is consistently depressor.

hemolysis distorted the spectrophotometer values were discarded.

## RESULTS

### *A. Effects of epinephrine and nor-epinephrine*

Epinephrine infusion produced systolic blood pressure elevation and usually a fall in the diastolic pressure. Even in the small dose employed in this study, subjects complained of a feeling of uneasiness, anxiety or nervousness. Many patients complained of palpitations and nearly all were aware of tachycardia. Pallor, cold sweat, and apprehension were frequently noted. Because of these unpleasant effects, higher infusion doses of epinephrine attempted during early studies were reduced.

Nor-epinephrine infusion uniformly produced a rise of systolic and diastolic blood pressure and fall in pulse rate with minimal subjective response. Patients appeared comfortable and relaxed even when the blood pressure was markedly elevated. Skin pallor was slight although constriction of the vein into which the infusion was running was usually apparent. Doses of nor-epinephrine two to three times greater than 0.085 μg. per Kg. per minute could be given with no discomfort if the dosage was increased gradually. When high doses were begun abruptly and blood pressure rose

sharply, severe headache was produced in several cases.

*B. Blood pressure and pulse responses*

*Histamine.* The effects of histamine were observed during nine infusions, eight of which were paired epinephrine and nor-epinephrine infusions in the same four subjects (see Figure 1). Histamine responses were consistently depressor during control periods (−20/−20 mm. Hg), during epinephrine infusion (−30/−20 mm. Hg), and also during nor-epinephrine infusion (−25/−20 mm. Hg). Pulse rate rose only slightly during controls (plus 5 beats per minute), during epinephrine (plus 3 beats per minute), and during nor-epinephrine infusion (plus 0.7 beats per minute). A slight flush followed by a moderately severe headache occurred regularly. These effects also were not significantly modified by epinephrine or nor-epinephrine infusion.

*Mecholyl®.* Mecholyl® effects were observed during seven infusions, six of which were paired epinephrine and nor-epinephrine infusions in the same three subjects (see Figure 2). Within 30 seconds after the drug was given subcutaneously, control pressures fell (−20/−25 mm. Hg) and

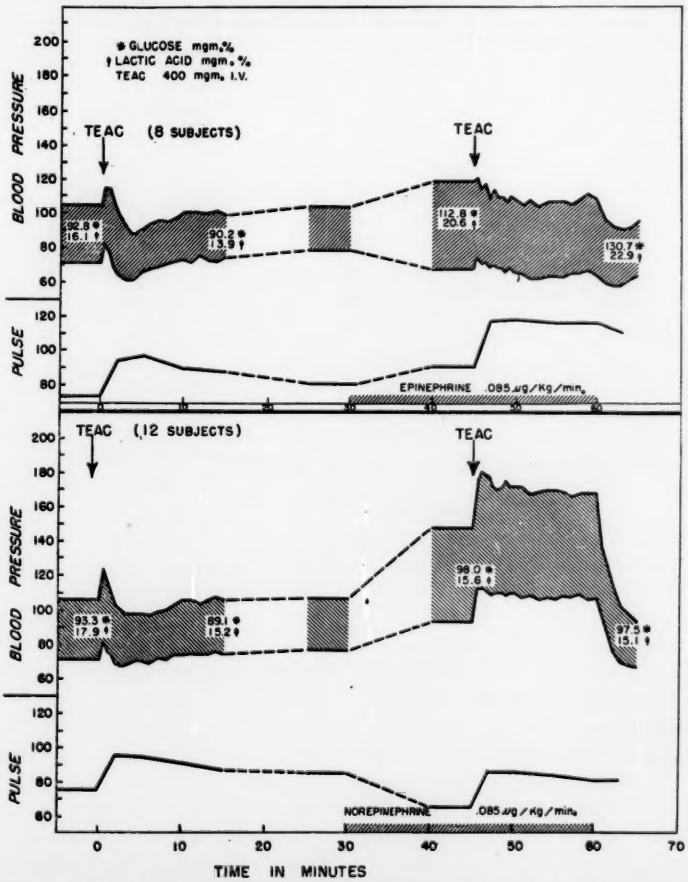



FIG. 3. MEAN RESPONSES TO TETRAETHYLAMMONIUM CHLORIDE (TEAC, 400 MG. INTRAVENOUSLY)

The data have been charted as in Figure 1. Note how the TEAC response becomes strikingly pressor during nor-epinephrine infusion (lower right).

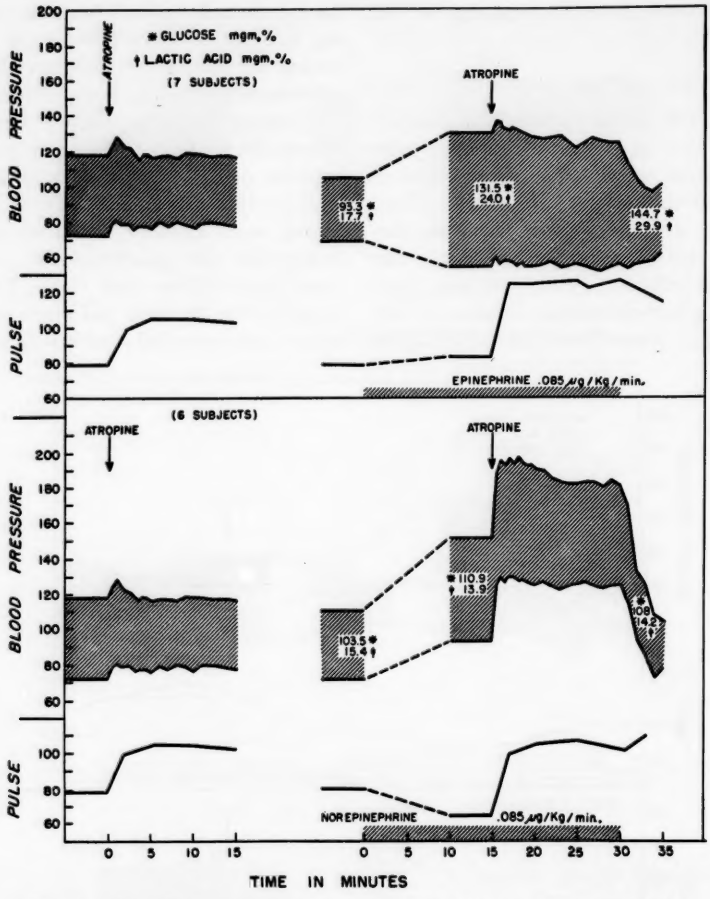



FIG. 4. MEAN RESPONSES TO ATROPINE SULFATE (1.2 MG. INTRAVENOUSLY)

The data are plotted as in Figure 1. As with TEAC, atropine causes marked potentiation of nor-epinephrine hypertension.

these depressor effects were even more prominent during epinephrine infusion (−30/−34 mm. Hg) and nor-epinephrine infusion (−48/−42 mm. Hg). Increase in pulse rate was more marked with Mecholyl® than with histamine, during control periods (plus 28 beats per minute), with epinephrine (plus 28 beats per minute), and during nor-epinephrine (plus 43 beats per minute). Tachycardia persisted at least 15 minutes. In addition, all patients exhibited the other muscarinic effects of Mecholyl®, such as flush, profuse sweating, salivation and an urge to defecate and urinate. Sweating was less profuse during epinephrine and nor-epinephrine infusion. There were no untoward side effects such as bronchospasm or cardiac arrhythmias.

*TEAC.* The response to TEAC was tested during 20 infusions, 8 epinephrine and 12 nor-epinephrine; 7 subjects received both (see Figure 3).

Control TEAC responses were only slightly depressor (−12/−8 mm. Hg) after an initial transient pressor rise lasting 30 to 60 seconds. TEAC responses during epinephrine infusions were also depressor (−15/−5 mm. Hg). During nor-epinephrine infusion, however, TEAC be-

came dramatically pressor (+33/+20 mm. Hg) and this persisted until the infusion was discontinued. The subjective response to the rapid injection of TEAC was usually a sudden metallic taste, followed by a generalized sensation of coolness and tingling in the hands and feet. Epinephrine and nor-epinephrine did not alter these reactions.

Pulse rate was increased by TEAC during the control periods (plus 23 beats per minute) and also during epinephrine (plus 27 beats per minute) and nor-epinephrine (plus 21 beats per minute).

*Atropine.* The response to atropine was tested during 13 infusions, 7 epinephrine and 6 nor-epinephrine, 6 subjects receiving both. During control tests with atropine blood pressures were essentially unchanged except for a transient slight increase in systolic and diastolic pressure (see Figure 4). During epinephrine infusion, atropine produced no significant change in blood pressure but during nor-epinephrine infusion blood pressure rose sharply (+45/+35 mm. Hg), and as with TEAC these potentiated pressor effects were sustained until the infusion was discontinued.

Atropine produced very little subjective effect. An occasional patient noted dryness of the mouth or palpitation. The pulse rate uniformly increased during the control test (plus 25 beats per minute), during epinephrine (plus 43 beats per minute), and during nor-epinephrine infusion (plus 41 beats per minute). In one patient paroxysmal auricular tachycardia was precipitated by atropine given during nor-epinephrine infusion.

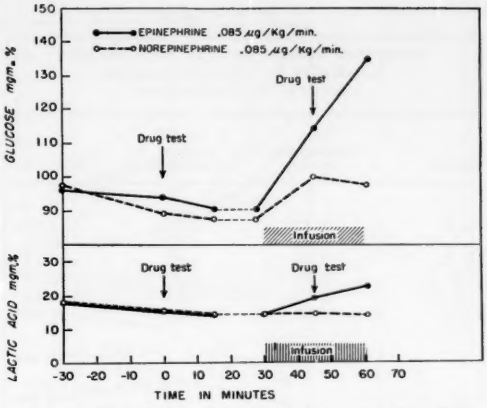



FIG. 5. THE MEAN GLUCOSE AND LACTIC ACID VALUES OBTAINED BEFORE AND AFTER CONTROL TESTS WITH HISTAMINE, MECHOLYL® AND TEAC (FAR LEFT) TOGETHER WITH THE CHANGES INDUCED BY EPINEPHRINE (SOLID LINE) AND NOR-EPINEPHRINE (DOTTED LINE)

It should be noted that these indices of adrenal medullary activity were not altered by the provocative test drugs.

*C. Metabolic studies*

Although the test drugs alone (histamine, Mecholyl®, and TEAC) had no effect on blood

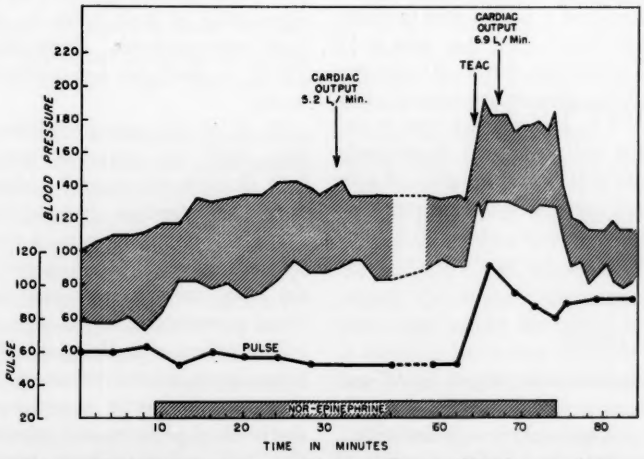



FIG. 6. DETERMINATION OF THE CARDIAC OUTPUT BEFORE AND AFTER POTENTIATION OF NOR-EPINEPHRINE BY TEAC

TABLE I

*Actual cardiac output values (liters per minute) during nor-epinephrine infusion and after blood pressure potentiation by tetraethylammonium chloride (TEAC) or atropine* *

| Subject | Nor-epinephrine infusion | Blood pressure potentiation | Difference† |
|---|---|---|---|
| | | TEAC | |
| K. B. | 6.22 | 8.10 | +1.88 |
| E. L. R. | 4.60 | 5.52 | +0.92 |
| W. R. | 5.16 | 6.88 | +1.72 |
| J. B. M. | 6.79 | 7.32 | +0.53 |
| J. L. W. | 4.87 | 6.27 | +1.40 |
| G. G. | 5.70 | 6.50 | +1.80 |
| | | Mean | +1.38 L./min. |
| | | S.E. | ±0.22 L./min. |
| | | | $0.01 > p > 0.001$ |
| | | Atropine | |
| T. G. | 4.59 | 8.04 | +3.45 |
| A. M. | 5.99 | 10.50 | +4.51 |
| O. L. B. | 4.56 | 5.76 | +1.20 |
| A. S. | 4.85 | 6.66 | +1.87 |
| J. W. | 5.74 | 7.10 | +1.36 |
| F. W. | 5.74 | 7.79 | +2.05 |
| | | Mean | +2.40 L./min. |
| | | S.E. | ±0.53 L./min. |
| | | | $0.01 > p > 0.001$ |

* Dye-dilution curve method of Hamilton and co-workers (8).
† Difference due to potentiation.

glucose or lactic acid levels (Figure 5) these were both elevated by epinephrine infusion, glucose rising from a mean of 90 mg. per cent to 114 mg. per cent during the first 15 minutes of infusion and then to a mean of 135 mg. per cent after 30 minutes of epinephrine. Lactic acid similarly rose from a mean of 14 to 19 mg. per cent in 15 minutes and to 22.5 mg. per cent in 30 minutes.

With nor-epinephrine, glucose level rose slightly from a mean of 87.5 to 100 mg. per cent in the first 15 minutes and did not change significantly (mean, 97.5 mg. per cent) in 30 minutes. Lactic acid levels were not changed by nor-epinephrine infusion nor were they affected when TEAC and atropine augmented the nor-epinephrine blood pressure effects.

### *D. Cardiac output studies*

To clarify the mechanism by which TEAC and atropine caused the observed sudden increases in blood pressure (nor-epinephrine potentiation), cardiac output was measured during a series of nor-epinephrine infusions before and approximately five minutes after potentiation by TEAC (15 subjects) or atropine (18 subjects) (see Figure 6 for a typical experiment).

In 12 experiments (6 TEAC and 6 atropine) satisfactorily paired curves were obtained and demonstrated a significant increase in cardiac output during potentiation ($p < 0.01$) (Table I). Potentiation by atropine increased cardiac output an average of 2.4 L. per minute ($p < 0.01$) and TEAC an average of 1.38 L. per minute ($p < 0.1$). Cardiac index (cardiac output divided by surface area in $M.^2$) increased 0.75 L. per minute per $M.^2$ with TEAC ($p < 0.01$) and 1.43 L. per minute per $M.^2$ with atropine ($p < 0.01$).

Pulse rate increased with TEAC (24 beats per minute) and with atropine (44 beats per minute) as in the other nor-epinephrine infusion experiments, and calculated stroke volume fell an average of 7 ml. per beat with TEAC potentiation and 8 ml. per beat with atropine, neither decrease being significant (Figure 7).

Calculated peripheral resistance (mean pressure times 1,332)/cardiac output did not change consistently with TEAC, decreasing in three papatients and increasing in three. However, with atropine peripheral resistance fell in all patients (mean fall, 198 units) ($p < 0.01$) (Figure 7).

### *E. Unusual responses*

In 6 of 33 infusions, TEAC failed to potentiate nor-epinephrine. In three of these the dose of nor-epinephrine in itself failed to change the control blood pressure levels. In the other three patients, TEAC in itself did not produce its usual tachycardia.

In 2 of 26 nor-epinephrine infusions atropine failed to potentiate nor-epinephrine. In both of these instances the blood pressure failed to rise with the dose of nor-epinephrine employed. In one of these cases when the dose of nor-epinephrine was doubled (0.170 $\mu$g. per Kg. per minute), the blood pressure rose and atropine then produced potentiation.

In a patient with Hodgkin's disease and a large tumor mass in the region of the right carotid sinus, atropine alone caused a marked, sustained rise in blood pressure and pulse and both epinephrine and nor-epinephrine were potentiated by atropine. This was the only patient in whom epi-

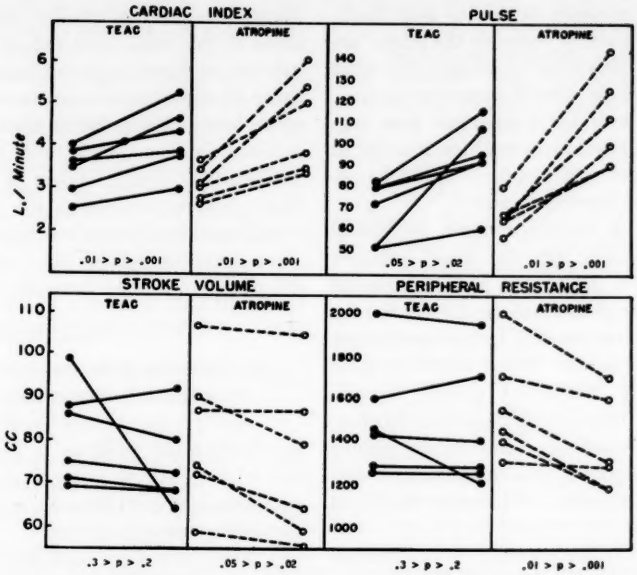



FIG. 7. THE VALUES FOR CARDIAC INDEX, PULSE, STROKE VOLUME AND PERIPHERAL RESISTANCE BEFORE (LEFT-HAND POINTS IN EACH BOX) AND DURING (RIGHT-HAND POINTS IN EACH BOX) NOR-EPINEPHRINE POTENTIATION BY TEAC (SOLID LINES) AND ATROPINE (BROKEN LINES)

nephrine was potentiated. After the tumor was shrunk with X-ray, epinephrine was no longer potentiated.

## DISCUSSION

It is apparent that of the several test drugs which induce pressor responses in cases of pheochromocytoma, TEAC must produce its effects by a different mechanism than histamine or Mecholyl®.

In these studies TEAC consistently potentiated the pressor effects of circulating nor-epinephrine. These responses closely resembled the paroxysmal blood pressure rises provoked by TEAC in pheochromocytoma and suggest that a positive provocative test with this drug constitutes an augmentation or potentiation phenomenon specific for circulating nor-epinephrine.

On the other hand, histamine and Mecholyl® were consistently depressor even in the presence of the infused pressor amines. Thus, a mechanism other than potentiation must account for their ability to induce pressor paroxysms.

Although it is speculative to compare our results in normal subjects during infusions to results in patients with pheochromocytoma, our subjects did resemble such patients in a very important way; namely, they had been made acutely hypertensive by circulating catecholamine.

The pharmacologic similarities of TEAC and atropine in these studies suggest that their ability to induce nor-epinephrine potentiation may be accounted for by a similar mechanism. Our data indicate that blood pressure augmentation is accompanied by an increase in cardiac output rather than by increase in peripheral resistance. This increase in output must be the result of a rise in heart rate, an increase in the force of cardiac contraction or both. Since neither TEAC nor atropine alone significantly increases cardiac output, the increase demonstrated with potentiation must reflect the action of these drugs (9) in speeding heart rate, tachycardia occurring at a time when the peripheral blood vessels are tightly constricted by nor-epinephrine.

This increase in pulse rate by TEAC and atropine almost certainly involves the buffer reflexes of the carotid sinus and the aortic arch, and the

augmented blood pressure responses both qualitatively and quantitatively resemble the acute "debuffering" effects observed by Kezdi (10) after Novocain® block of the carotid sinuses in man.

In accordance with the mechanism first suggested by Moe (11) and Hoobler, Moe, and Lyons (9), potentiation as observed here might be accounted for in the following manner.

With infusion of nor-epinephrine, peripheral vasoconstriction occurs and the blood pressure rises. The carotid sinus and aortic depressor nerve are stimulated to increase vagal tone, thereby slowing the heart and tending to prevent further rise of the blood pressure. When TEAC or atropine are suddenly introduced during nor-epinephrine infusion, the vagal bradycardia is blocked, heart rate increases and any compensatory change in peripheral resistance is prevented by (nor-epinephrine) vasoconstriction. Therefore the blood pressure must rise.

The failure to produce potentiation of epinephrine suggests a significant difference between man and the experimental animal, since Page and Taylor (12), Moe (11), and Goldblatt (13) have observed potentiated epinephrine responses to TEAC in animals.

Our inability to produce epinephrine potentiation in man may reflect: 1) epinephrine's vasodilating effect in contrast to nor-epinephrine's vasoconstriction (14); 2) that the dose of epinephrine tolerated by man may be too low to permit potentiation; or 3) that the cardiac output may already be maximally increased before the potentiating agent is introduced.

Because of the striking potentiation of nor-epinephrine by atropine in these studies, we have employed this drug as a provocative test for pheochromocytoma four times in three patients with proven tumors. On two occasions the blood pressure rose sharply after 1.2 mg. of intravenous atropine but on the other two the blood pressure remained at control levels in spite of the appearance of marked tachycardia. Since these patients had consistently pressor TEAC responses, it is suggested that other mechanisms beyond simple buffer block may participate in the positive provocative test with TEAC.

The blood sugar and lactic acid data confirm the known differences in the metabolic effects of nor-epinephrine and epinephrine infusion (15). In the present study, histamine, Mecholyl® or TEAC alone in the doses used did not cause significant increase in blood sugar or lactic acid to indicate firing of the adrenal medulla with elaboration of epinephrine. Since it has been shown (16) that increase in blood sugar and lactic acid can be produced by smaller doses of epinephrine than those required to change the blood pressure, it would seem that histamine, Mecholyl® and TEAC given intravenously in the control studies here did not stimulate the adrenals.

## CONCLUSIONS

The results suggest the following conclusions:

1. Since tetraethylammonium chloride (TEAC) consistently augments the hypertension induced by nor-epinephrine infusion a positive provocative test with TEAC in pheochromocytoma may well represent a potentiation phenomenon specific for circulating nor-epinephrine.

2. Since histamine and Mecholyl® remain depressor during epinephrine and nor-epinephrine infusion, a different mechanism must account for the pressor responses which these agents may induce in pheochromocytoma.

3. In contrast to the results in the experimental animal epinephrine potentiation could not be produced in man with TEAC.

4. Atropine, like TEAC, potentiates nor-epinephrine and deserves further evaluation as a screening agent for pheochromocytoma.

5. Pressor potentiation of nor-epinephrine by TEAC and atropine is accompanied by an increase in cardiac output and no significant change in peripheral resistance, probably reflecting blockade of the carotid sinus and aortic arch reflexes which control heart rate.

6. Metabolic studies produced no evidence to suggest that histamine, Mecholyl® or TEAC in the doses employed provoked stimulation of the normal adrenal medulla.

## ACKNOWLEDGMENT

The authors are indebted to Miss Leona Witherow and Miss Collette Barbin for valuable technical assistance in the conduct of these studies.

## REFERENCES

1. Roth, G., and Kvale, W. F. A tentative test for pheochromocytoma. Amer. J. med. Sci. 1945, **210**, 653.

2. Guarneri, V., and Evans, J. A. Pheochromocytoma, report of a case, with a new diagnostic test. Amer. J. Med. 1948, **4**, 806.
3. La Due, J. S., Murison, P. J., and Pack, G. T. The use of tetraethylammonium bromide as a diagnostic test for pheochromocytoma. Ann. intern. Med. 1945, **29**, 914.
4. Littman, A., Grossman, M. I., Gunnar, R. M., Isaacs, J. H., Hirschmann, J. H., and Foley, E. F. Electrocardiographic effects of arterenol and isopropylarterenol in man, with a note on the auricular T wave. J. appl. Physiol. 1950, **3**, 235.
5. Littman, A., Gunnar, R. M., Grossman, M. I., and Casas, R. Effect of posture on hypertension induced by sympathomimetic amines in man. Circulation 1952, **5**, 437.
6. Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. J. biol. Chem. 1944, **153**, 375.
7. Hawk, P. B., Oser, B. L., and Summerson, W. H. Practical Physiological Chemistry, 12th ed. Philadelphia, The Blakiston Company, 1947.
8. Hamilton, W. F., Riley, R. L., Attyah, A. M., Cournand, A., Fowell, D. M., Himmelstein, A., Noble, R. P., Remington, J. W., Richards, D. W., Jr., Wheeler, N. C., and Witham, A. C. Comparison of the Fick and dye injection methods of measuring the cardiac output in man. Amer. J. Physiol. 1948, **153**, 309.
9. Hoobler, S. W., Moe, G. K., and Lyons, R. H. The cardiovascular effects of tetraethylammonium in animals and man with special references to hypertension. Med. Clin. N. Amer. 1949, **33**, 805.
10. Kezdi, P. Sinoaortic regulatory system. Arch. intern. Med. 1953, **91**, 26.
11. Moe, G. K. Potentiation of pressor action of epinephrine by tetraethyl ammonium. J. Amer. med. Ass. 1948, **137**, 1115.
12. Page, I. H., and Taylor, R. D. Sensitization to the pressor action of epinephrine ("adrenalin"). J. Amer. med. Ass. 1947, **135**, 348.
13. Goldblatt, H. In discussion. Proc. Ann. Meeting Soc. High Blood Pressure Research 1955, **4**, 60.
14. Goldenberg, M., Pines, K. L., Baldwin, E. de F., Greene, D. G., and Roh, C. E. The hemodynamic response of man to nor-epinephrine and epinephrine and its relation to the problem of hypertension. Amer. J. Med. 1948, **5**, 792.
15. DiSalvo, R. J., Bloom, W. L., Brust, A. A., Ferguson, R. W., and Ferris, E. B. A comparison of the metabolic and circulatory effects of epinephrine, nor-epinephrine and insulin hypoglycemia with observations on the influence of autonomic blocking agents. J. clin. Invest. 1956, **35**, 568.
16. Von Euler, U. S., and Ström, G. Present status of diagnosis and treatment of pheochromocytoma. Circulation 1957, **15**, 5.

# A STUDY OF CARDIOPULMONARY ALTERATIONS IN PATIENTS WITH SICKLE CELL DISEASE AND ITS VARIANTS [1]

By BRIAN J. SPROULE, E. RICHARD HALDEN, AND WILLIAM F. MILLER

*(From the Cardiopulmonary Laboratory, Department of Internal Medicine, University of Texas Southwestern Medical School, and Parkland Memorial Hospital, Dallas, Texas)*

(Submitted for publication September 3, 1957; accepted November 21, 1957)

It has recently become apparent that a high proportion of individuals who possess S type hemoglobin exhibit significant desaturation of the arterial blood. In 1942 Klinefelter (1) found arterial oxygen saturations of 89 and 91 per cent in two patients, and in 1943 Reinhard, Moore, Dubach, and Wade (2) found values ranging from 74.2 to 90.8 per cent saturation in one patient with sickle cell disease. Leight, Snider, Clifford, and Hellems (3) studied 13 patients with sickle cell disease in 1954, 10 of whom had arterial $O_2$ desaturation. Within the past year reports from this laboratory (4, 5) and from Jensen, Rucknagel, and Taylor (6) indicate that desaturation of arterial blood exists in most people who possess S type hemoglobin. The exact mechanism responsible for this phenomenon has not been elucidated.

The various possibilities may be distinguished with fair accuracy by determining alveolar and arterial oxygen tensions and calculating A-a oxygen tension gradients at three levels of oxygenation. If desaturation is due to hypoventilation, no appreciable gradient exists at any level of oxygenation and $CO_2$ retention is usually evident. Any increase in A-a gradient must, therefore, be due to some other mechanism. Lilienthal, Riley, Proemmel, and Franke (7) suggested that one might distinguish between a diffusion defect and veno-arterial admixture by measurement of the effective alveolar-arterial gradient at two levels of oxygenation, one of which produces significant desaturation of arterial blood. Low levels of inspired $O_2$ (12 to 14 per cent) exaggerate the effects of impaired diffusion and minimize the effects of veno-arterial admixture. Veno-arterial admixture, thus delineated, may be either through actual anatomical communications, or the result of reduction in the ventilation-perfusion ratios. If due to uneven ventilation, the gradient is eliminated by administration of 100 per cent oxygen since, during pure oxygen breathing, even minimally ventilated alveoli eventually attain an oxygen tension approaching atmospheric pressure. Therefore, as shown by Berggren (8), the A-a gradient measured during the inspiration of pure oxygen may be taken as a measure of shunting of blood from the venous to the arterial side of the circulation.

In the following studies, these concepts were utilized in an effort to clarify the mechanism whereby desaturation occurs in individuals with sickle cell anemia and its variants. In addition, observations were made on the hemodynamic alterations accompanying cardiomegaly in these states and on the relationship of intravascular oxygen tension and degree of sickling to the sickle cell crisis.

## MATERIAL AND METHODS

Alveolar-arterial oxygen gradients were measured during the inspiration of low oxygen, room air, and 100 per cent oxygen on 21 subjects possessing S type hemoglobin, 4 of whom had hemoglobin electrophoretic patterns characterized as SA (sickle cell trait), 4 as SC (sickle cell hemoglobin C disease), and 13 as SS (sickle cell disease). The 0.5 second timed expiratory capacities and vital capacities were measured on all subjects using a Gaensler-Collins vitalometer. Cardiac catheterization was done in 10 members of the group. Quantitation of the *in vivo* sickling phenomenon on both the arterial and venous sides of the circulation, along with measurement of serum hemoglobin levels, was also undertaken.

All measurements were made in the supine position. No premedication was routinely given although the 10 patients who underwent cardiac catheterization received procaine amide hydrochloride (Pronestyl®), 250 mg., and penicillin, 600,000 units, on the morning of the studies.

Arterial blood collections were made from an indwelling Cournand needle inserted into the brachial artery; venous blood samples were obtained from peripheral veins in both arms. Gas mixtures were breathed through a Rudolph two-way breathing valve with Bennett-seal mouthpiece, and expired air was collected in a Douglas

[1] This work is part of a project being supported by a grant from the United States Public Health Service (H-2113).

bag. Arterial blood was analyzed for $CO_2$ and $O_2$ content, pH, $O_2$ tension ($pO_2$), per cent sickling, and quantity of serum hemoglobin. Venous blood was analyzed for $pO_2$ or $O_2$ content, or both, per cent sickling, and serum hemoglobin. Venous pH was determined in the blood samples analyzed for $O_2$ content, and $O_2$ tensions were calculated using these values, and the $O_2$ dissociation curves of Dill (9) in those patients from whom directly measured venous $O_2$ tensions were not obtained. Carbon dioxide and $O_2$ contents were determined by the method of Van Slyke and Neill, duplicate determinations being required to check within 0.1 volumes per cent. Hydrogen ion concentration was measured at 37.5° C. on a Cambridge research model pH meter. On the first few patients $pO_2$ values were determined using a polarograph with a dropping mercury electrode for high $O_2$ tension (> 100 mm. Hg) and the microbubble equilibration of Riley, Proemmel, and Franke (10) for physiologic $O_2$ tensions. Later, a new polarographic technique employing the Clark electrode was utilized to measure $O_2$ tensions at all levels. The accuracy and reliability of the method at all levels of $O_2$ tension have been established by a comparison between directly measured $O_2$ tensions and those calculated from simultaneously drawn samples of blood analyzed on the Van Slyke manometric apparatus (11). It was found that, over a $pO_2$ range of 23 to 97 mm. Hg, the relation between calculated and polarographically determined values was very nearly linear, the correlation coefficient being + 0.98 (n = 57). Comparison over the same range of $pO_2$ between the microbubble technique of Riley and the polarographic method yields similar results. At higher levels of $pO_2$, blood was equilibrated with tank mixtures, the $pO_2$ of which varied from 133 to 710 mm. Hg as determined from Scholander analyses (12). Again, the relationship between the known $pO_2$ and that determined polarographically was linear, variations between individual pairs being negligible.

Blood for analysis of per cent sickling was withdrawn anaerobically into Zenker's solution and stained with safranin, utilizing a technique described elsewhere (13). Counts were done on 1,000 to 1,500 cells. Serum hemoglobin was determined using Crosby's modification of the benzidine method (14).

Expired air was analyzed for $O_2$ content on a Beckman E-2 magnetic $O_2$ analyzer and for $CO_2$ content on a Liston-Becker infrared $CO_2$ analyzer. These instruments were standardized daily with known gas mixtures and were checked periodically with a Scholander microanalyzer. Gas volumes were measured with a Tissot spirometer and were corrected either to BTPS or to STPD. Physiological dead space was calculated on all patients using a conventional equation (15).

As soon as the subject appeared to have attained a steady state, a gas collection was made, blood samples being withdrawn midway during the collection. The per cent of oxygen in inspired air was checked on the inspiratory side of the breathing valve with the Beckman $O_2$ analyzer during the collection of expired gas.

On completion of the ambient air measurements, a low oxygen mixture was administered for 30 minutes; all measurements were then repeated. Since we were critically concerned with the effects of low $pO_2$ on the *in vivo* sickling phenomenon, the levels of $O_2$ used (9 to 15 per cent) were occasionally lower than those recommended by Riley and Cournand (16).

One hundred per cent $O_2$ was then administered for the same length of time, at the end of which the blood and gas collections were once again repeated. Adequate washout of $N_2$ by the 100 per cent $O_2$ was periodically checked by monitoring expired air on a nitrogen meter.

The alveolar-arterial gradients for $O_2$ were determined using the alveolar air equation to calculate the alveolar $pO_2$. Carbon dioxide tension for use in this equation was derived from the measured arterial $CO_2$ content (Van Slyke), and pH by use of the Singer-Hastings nomogram (17). The directly measured (polarographic) value for arterial $pO_2$ was then subtracted from the calculated alveolar $pO_2$. To use the A-a gradient obtained in this way for calculation of the approximate magnitude of veno-arterial admixture, it is necessary to perform the determinations while 100 per cent $O_2$ is being breathed and also to know the AV $O_2$ difference (18). In the 10 catheterized patients, this was measured directly. In eight additional patients, cardiac outputs were estimated at all levels of oxygenation by a modification of a $CO_2$ method described by Forssander (19). Comparisons between cardiac outputs determined in this way with those determined simultaneously by the conventional Fick method showed a systematic discrepancy for which a correction factor was worked out. The corrected cardiac output could then be used along with the $O_2$ consumption to calculate AV $O_2$ difference. In the 10 remaining determinations an AV $O_2$ difference of 3.75 volumes per cent (which was the average of the foregoing) was assumed. The magnitude of right-to-left shunts may be estimated as follows (18):

$$\text{Per cent shunt} = 100 - (Y/Y\ \&\ G) \times 100,$$

where Y = AV $O_2$ difference, G = Gradient in mm. Hg times 0.003 (Sendroy factor to convert dissolved $O_2$ at 37.5° C. to volumes per cent).

Right heart catheterization, when done, was performed in the conventional manner. A wedge pressure was recorded and the catheter was then withdrawn and left in the pulmonary artery during gas exchange studies. Pressures were recorded by means of a pressure transducer and a multichannel recorder. Dye curves were obtained using an ear oximeter, following the injection of T-1824 dye into the right atrium.

Radioactive chromium blood volumes were done on seven of the catheterized patients.

## RESULTS

### *A. Room air breathing*

Arterial $O_2$ desaturation was present in 16 of the 21 patients (Table I), the mean arterial $O_2$ saturation being 89.1 per cent, the mean arterial

TABLE I

*Results on room air breathing—Desaturated subjects* *

| Patient No. | Age | Hb. pattern | Hematocrit | Hb. *Gm.* | Brachial artery | | | Peripheral venous | | Pulmonary artery | | A-a gradient | Pulmonary function data‡ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | Vitalometry | | |
| | | | | | Sat. % | $pO_2$ | % Sickle | $pO_2$† | % Sickle | $pO_2$ | % Sickle | | 0.5 sec. EC / VC ×100 % | VC / PVC ×100 % | Physiolog. dead space *ml.* |
| 1. J. A. | 35 | SS | 14 | 4.9 | 90.2 | 66 | 6.0 | 70 | 5.0 | | | 42 | 72 | 37 | 138.8 |
| 2. J. A. | | | 34 | 12.3 | 79.6 | 49 | 0.0 | 46 | 0.0 | 36 | 0.0 | 54 | 71 | 44 | 117.2 |
| 3. E. R. | 20 | SS | 17 | 7.8 | 85.0 | 60 | 21.0 | 41 | 28.0 | | | 45 | | | 146.5 |
| 4. E. R. | | | 19 | 8.1 | 87.0 | 61 | 14.5 | 58 | 17.7 | 35 | 26.3 | 34 | 55 | 65 | 142.4 |
| 5. W. Y. | 14 | SS | 18 | 7.8 | 85.0 | 52 | 9.6 | | 56.0 | | | 47 | | | 101.8 |
| 6. W. Y. | | | 21 | 7.1 | 86.7 | 60 | 12.3 | 44 | 25.7 | 36 | 40.9 | 28 | 74 | 68 | 91.3 |
| 7. R. C. R. | 17 | SS | 23 | 8.4 | 87.9 | 50 | 24.1 | 24 | 45.9 | | | 47 | 71 | 66 | 103.7 |
| 8. R. C. R. | | | 26 | 8.6 | 88.0 | 52 | 14.2 | 38 | 29.2 | 43 | 29.3 | 47 | 73 | 65 | |
| 9. K. G. | 35 | SS | 21 | 7.9 | 91.1 | 67 | 10.3 | 51 | 3.9 | | | 36 | 71 | 62 | 120.8 |
| 10. K. G. | | | 22 | 7.8 | 90.6 | 64 | 9.6 | 55 | 41.2 | 33 | 60.0 | 32 | | | |
| 11. R. T. | 30 | SS | 33 | 9.6 | 87.6 | 56 | 12.9 | 41 | 15.6 | | | 34 | 62 | 75 | 151.2 |
| 12. E. D. | 36 | SS | 24 | 8.0 | 87.5 | 58 | 11.8 | 56 | 12.9 | 49 | 20.3 | 46 | 74 | 68 | |
| 13. T. P. | 23 | SS | 24 | 8.8 | 88.9 | 65 | 10.7 | 45 | 18.0 | | | 34 | 64 | 65 | 78.7 |
| 14. R. W. | 33 | SS | 28 | 9.2 | 91.1 | 64 | 11.8 | 26 | 29.4 | | | 35 | 68 | 73 | 186.9 |
| 15. C. J. | 20 | SS | 27 | 9.0 | 92.8 | 78 | 9.8 | 36 | 22.4 | | | 18 | 68 | 64 | 185.5 |
| 16. C. P. | 45 | SS | 33 | 10.6 | 88.6 | 66 | 7.2 | 45 | 42.0 | | | 37 | 76 | 62 | 157.7 |
| 17. W. S. | 26 | SA | 33 | 11.6 | 92.0 | 62 | 2.9 | 61 | 2.9 | | | 38 | 68 | 61 | 219.3 |
| 18. S. A. | 27 | SA | 32 | 10.9 | 94.3 | 80 | 2.9 | 25 | 4.0 | | | 20 | 66 | 98 | 104.0 |
| 19. E. M. | 26 | SA | 32 | 10.8 | 93.7 | 70 | 3.0 | | 3.0 | | | 25 | 64 | 71 | 53.7 |
| 20. B. T. | 35 | SC | 27 | 10.0 | 92.5 | 78 | 2.1 | 21 | 3.6 | | | 34 | 59 | 62 | 104.0 |
| 21. J. M. | 43 | SC | 26 | 10.5 | 91.8 | 70 | 2.8 | 34 | 5.6 | | | 24 | 65 | 57 | 121.4 |
| Mean | | | 25.4 | 9.03 | 89.13 | 63.23 | 9.5 | 43.0 | 19.6 | 38.6 | 29.4 | 36.1 | 67.8 | 64.6 | 129.15 |
| S. D. | | | ±5.96 | ±1.68 | ±3.59 | ±8.7 | ±6.0 | ±13.2 | ±16.2 | ±6.0 | ±18.1 | ±9.4 | ±5.8 | ±12.2 | ±40.12 |

* Desaturation defined as an A-a gradient > 15 mm. Hg.
† Abnormally high venous $pO_2$ determinations—Nos. 1, 2, 4, 9, 12, 17.
‡ EC = Expiratory capacity; VC = Vital capacity; PVC = Predicted vital capacity.

oxygen tension 63.2 mm. Hg, and the average A-a gradient 36.0 mm. Hg. Arterial saturation was normal in six patients (Table II) who had a mean arterial saturation of 96.6 per cent, mean oxygen tension of 90.7 mm. Hg, and an average A-a gradient of 9.6 mm. Hg. It might be noted that Patient No. 21, J. M., moved from the desaturated to the saturated group when repeat studies were performed after an interval of two months. The total of desaturated plus saturated subjects therefore sums to 22 rather than 21 since Patient J. M. is counted twice by this method. All three types of hemoglobin electrophoretic patterns were found in both the desaturated (11 SS, 3 SA, and 2 SC) and the normally saturated (2 SS, 2 SC, and 1 SA) groups. The mean hemoglobin of the normally saturated subjects was 10.18 Gm. The per cent of sickling in venous and arterial blood was directly related to the $pO_2$; the levels of $pO_2$ required to produce sickling, however, differed according to the hemoglobin variant (SA, SC, or SS) (4). The venous oxygen tensions varied greatly; abnormally high readings, closely approximating simultaneously drawn arterial oxygen tensions, appeared in numerous instances. Values for oxygen tension in determinations Nos. 6, 7, 9, 11, 15, 17, 21, and 25 were derived from $O_2$ content (Van Slyke) and pH. Of these, determinations Nos. 9 and 17 demonstrated directly the abnormally high $O_2$ content suggested by polarographic measurement of $O_2$ tension in a number of other patients (Nos. 1, 2, 4, and 12). Simultaneously drawn pulmonary artery blood was significantly lower in oxygen tension than the arterialized peripheral venous blood.

### *B. Low oxygen breathing*

In Table III are listed the results obtained when the same patients were evaluated after breathing a low concentration of oxygen for 30 minutes. This phase of the complete study was usually omitted on repeat determinations, but in order to facilitate comparisons, numbers corresponding to Tables I and II have been retained. The average gradient in the desaturated subjects (determinations Nos. 1 to 21) was 14.0 mm. Hg as compared to a gradient of 9.4 mm. Hg in the completely saturated individuals. Without exception, the A-a gradient was less than the room air values when

TABLE II

*Results on room air breathing—Normally saturated subjects**

| Patient No. | Age | Hb. pattern | Hemato-crit | Hb. Gm. | Brachial artery | | | Peripheral venous | | Pulmonary artery | | A-a gradient | Pulmonary function data† | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Sat. % | $pO_2$ | % Sickle | $pO_2$ | % Sickle | $pO_2$ | % Sickle | | Vitalometry 0.5 sec. EC VC ×100 % | Vitalometry VC PVC ×100 % | Physiolog. dead space ml. |
| 22. J. M. | | | 27 | 10.6 | 94.1 | 80 | | 32 | 33.0 | 37 | 9.9 | 12 | 60 | 62 | 180.3 |
| 23. H. M. | 17 | SC | 29 | 9.0 | 95.6 | 90 | 3.2 | 34 | 10.0 | | | 13 | 58 | 84 | 179.0 |
| 24. H. M. | | | 31 | 9.3 | 96.1 | 87 | 3.0 | 60 | 5.2 | 45 | 6.2 | 8 | | | 118.7 |
| 25. A. D. | 42 | SC | 28 | 12.1 | 95.4 | 90 | 1.1 | 32 | 9.8 | 44 | 9.8 | 12 | 62 | 95 | 181.8 |
| 26. R. C. | 38 | SA | 41 | 12.2 | 99.1 | 100 | | | | 40 | | 0 | 53 | 73 | 86.9 |
| 27. M. M. | 38 | SS | 28 | 9.5 | 97.1 | 88 | 7.8 | 30 | 18.8 | | | 12 | 67 | 79 | 69.0 |
| 28. K. M. J. | 22 | SS | 24 | 8.6 | 98.8 | 100 | 8.5 | 60 | 8.7 | | | 9 | 81 | 74 | 128.8 |
| Mean | | | 31.1 | 10.18 | 96.6 | 90.7 | 4.7 | 41.3 | 14.2 | 43.0 | 8.6 | 11.0 | 63.5 | 77.8 | 134.93 |
| S. D. | | | ±5.9 | ±1.41 | ±1.7 | ±10.5 | ±2.9 | ±13.3 | ±9.4 | ±13.2 | ±1.9 | ±1.8 | ±8.8 | ±10.4 | ±43.34 |

* Normal saturation defined as being present if the A-a gradient is <15 mm. Hg.
† EC = Expiratory capacity; VC = Vital capacity; PVC = Predicted vital capacity.

TABLE III

*Results on low $O_2$ breathing*

| Patient No. | Hb. electrophoretic pattern | $O_2$ in inspired gas (%) | Arterial | | | Venous | | A-a $O_2$ gradient (*mm. Hg*) |
|---|---|---|---|---|---|---|---|---|
| | | | Sat. (%) | $pO_2$ (*mm. Hg*) | % Sickle | $pO_2$ (*mm. Hg*)* | % Sickle | |
| 1. J. A. | SS | 15.9 | 78.0 | 43 | 7.0 | | 7.0 | 30.0 |
| 3. E. R. | SS | 11.7 | 47.0 | 29 | 56.0 | 36 | 69.2 | 29.0 |
| 5. W. Y. | SS | 11.6 | 68.0 | 40 | 44.5 | | 19.0 | 8.4 |
| 7. R. C. R. | SS | 9.2 | 50.8 | 30 | 37.0 | 14 | 75.6 | 5.0 |
| 9. K. G. | SS | 11.1 | 54.4 | 31 | 45.7 | 13 | 63.0 | 13.6 |
| 11. R. T. | SS | 14.1 | 73.0 | 34 | 29.2 | 37 | 33.8 | 25.0 |
| 12. E. D. | SS | 16.0 | 82.0 | 46 | 15.4 | 49 | 20.0 | 28.0 |
| 13. T. P. | SS | 8.9 | 54.9 | 28 | 41.0 | 12 | 70.6 | 4.0 |
| 14. R. W. | SS | 10.5 | 51.0 | 28 | 40.1 | 19 | 57.9 | 11.0 |
| 15. C. J. | SS | 11.1 | 80.0 | 46 | 11.6 | 20 | 40.8 | 0.0 |
| 16. C. P. | SS | 10.4 | 51.8 | 28 | 40.6 | 10 | 62.3 | 12.0 |
| 17. W. S. | SA | 11.1 | 66.4 | 29 | 16.3 | 37 | 6.5 | 10.0 |
| 18. S. A. | SA | 9.5 | 60.0 | 30 | 3.1 | 13 | 25.0 | 12.0 |
| 19. E. M. | SA | 10.9 | 60.0 | 29 | 3.0 | 18 | 3.0 | 13.0 |
| 20. B. T. | SC | 10.2 | 69.7 | 31 | 12.1 | 22 | 15.0 | 11.5 |
| 21. J. M. | SC | 11.5 | 54.4 | 28 | 27.0 | 18 | 50.0 | 11.4 |
| Mean | | | 62.4 | 33.1 | | | | 14.0 |
| S. D. | | | ±3.5 | ±6.9 | | | | ±8.8 |
| 23. H. M. | SC | 11.4 | 68.0 | 34 | 16.0 | 25 | 21.0 | 13.0 |
| 25. A. D. | SA | 14.5 | 87.0 | 55 | 2.4 | 35 | 4.6 | 11.0 |
| 26. R. C. | SA | 17.6 | 94.8 | 82 | | | | 8.0 |
| 27. M. M. | SS | 12.9 | 68.0 | 34 | 18.1 | 24 | 32.3 | 12.0 |
| 28. K. M. J. | SS | 9.6 | 50.1 | 40 | 55.8 | 42 | 38.3 | 3.0 |
| Mean | | | 73.6 | 49 | | | | 9.4 |
| S. D. | | | ±16.02 | ±18 | | | | ±3.6 |

* Abnormally high venous $pO_2$'s—Nos. 3, 11, 12, 17, 28.

low oxygen was breathed. Peculiar variations in the venous $pO_2$ were again seen. On five occasions, the venous $pO_2$ was higher than simultaneously determined arterial $pO_2$. In determination No. 17 this abnormality was demonstrated by simultaneous measurement of arterial and venous $O_2$ content, which has, in Table III, been converted to venous $O_2$ tension by use of pH and the $O_2$ dissociation relationship. Venous blood was found to contain fewer sickled cells than arterial blood (inferentially suggesting a higher $pO_2$ in venous than in arterial blood) on three occasions.

### *C. One hundred per cent oxygen breathing*

The values obtained when the patients breathed 100 per cent $O_2$ (Table IV) indicate that a significant degree of right-to-left shunting existed in most of these subjects. The individuals who exhibited arterial $O_2$ desaturation while breathing room air had, while breathing pure oxygen, an average A-a gradient of 165.4 mm. Hg with a calculated shunt of 11.96 per cent. That desaturation is related to shunting is suggested by the fact that the six normally saturated individuals, when studied with pure oxygen, showed an average A-a gradient of only 28.3 mm. Hg with a calculated shunt of 1.93 per cent. One patient (J. A., Nos. 1 and 2) showed a gross increase in the degree of shunting when her hemoglobin was increased from 5.0 to 11.0 Gm. by transfusion. Measurement of venous $pO_2$ in a number of cases showed evidence of marked arterialization, which became more obvious during 100 per cent oxygen breathing than at lower levels.

### *D. Cardiac catheterization data*

Hemodynamic data on 10 patients are set out in Table V. (The numbers identify the patients as listed in previous tables.) With the exception of Subject No. 26, all had radiologic evidence of cardiomegaly. In all subjects the pulmonary vascular resistance was within normal limits. The average cardiac indexes were appreciably elevated and the total blood volumes, done by the radio-

active chromium method, were increased in all desaturated subjects. The appearance time of all T-1824 dye curves was normal.

### *E. Additional data*

The results of analysis of blood samples obtained simultaneously from two peripheral veins, the brachial artery and, in two instances, from the pulmonary artery, are presented in Table VI. These data offer further evidence of abnormally high oxygen contents in certain peripheral veins.

The ratio (0.5 sec. EC/VC) × 100 is indicative of a significant obstructive ventilatory defect if less than 60 per cent; vital capacity is indicative of a significant restrictive ventilatory defect if it is less than 85 per cent of the predicted normal (20). The desaturated subjects were found to have an average (0.5 second EC/VC) ratio of 67.8 ± 5.8 per cent, with an average vital capacity of 64.6 ± 12.2 per cent of predicted normal. These values are suggestive of a pure restrictive ventilatory defect. Timed vitalometry on the normally saturated individuals showed virtually normal values.

The average physiological dead space was within normal limits in both the desaturated (129.15 ± 40.21 ml.) and the normally saturated (134.93 ± 43.34 ml.) subjects.

Serum hemoglobins were unaltered by the inhalation of various oxygen mixtures and were also unchanged when measured on 10 of these patients during periods of clinical "crises."

## DISCUSSION

### *Arterial oxygen desaturation*

These results confirm the existence of desaturation of the arterial blood in a majority of subjects possessing the abnormal S type of hemoglobin. The phenomenon is not clearly related either to the specific hemoglobin electrophoretic pattern or to the degree of associated anemia.

TABLE IV

*Results on 100 per cent $O_2$ breathing*

| Patient No. | Hb. electrophoretic pattern | Arterial* | | Venous | | A-a gradient (*mm. Hg*) | Calc. % shunt |
|---|---|---|---|---|---|---|---|
| | | $pO_2$ (*mm. Hg*) | % Sickle | $pO_2$ (*mm. Hg*) | % Sickle | | |
| 1. J. A. | SS | 542 | 1.6 | 48 | 3.1 | 108 | 8.3 |
| 2. J. A. | | 191 | | 83 | | 457 | 27.4 |
| 4. E. R. | SS | 374 | 10.5 | 300 | 10.5 | 290 | 27.0 |
| 5. W. Y. | SS | 411 | 3.2 | 300+ | 45.0 | 257 | 17.6 |
| 7. R. C. R. | SS | 367 | 7.1 | 60 | 24.0 | 239 | 14.2 |
| 8. R. C. R. | | 470 | 6.2 | 88 | 3.7 | 208 | 17.4 |
| 9. K. G. | SS | 528 | 1.6 | 339 | 2.2 | 145 | 8.8 |
| 11. R. T. | SS | 596 | 2.3 | 256 | 2.5 | 79 | 6.8 |
| 12. E. D. | SS | 482 | 1.2 | 308 | 1.3 | 194 | 16.0 |
| 13. T. P. | SS | 550 | 5.4 | 319 | 0.9 | 112 | 9.4 |
| 14. R. W. | SS | 519 | 1.8 | 50 | 8.7 | 145 | 9.4 |
| 15. C. J. | SS | 400 | 0.4 | 120 | 2.2 | 264 | 16.6 |
| 16. C. P. | SS | 516 | 2.3 | 53 | 4.4 | 152 | 11.0 |
| 17. W. S. | SA | 504 | 2.2 | 30 | 11.9 | 155 | 11.4 |
| 18. S. A. | SA | 584 | 2.4 | 71 | 3.2 | 89 | 6.9 |
| 19. E. M. | SA | 538 | 3.0 | 41 | 3.0 | 129 | 9.6 |
| 20. B. T. | SC | 501 | 0.4 | 36 | | 162 | 11.9 |
| 21. J. M. | SC | 344 | 0.2 | 57 | 10.0 | 290 | 11.0 |
| Mean | | 400.8 | | | | 165.4 | 11.5 |
| S. D. | | ±187.2 | | | | ±108.0 | ±7.2 |
| 22. J. M. | | 606 | 2.2 | 50 | 16.7 | 66 | 4.4 |
| 24. H. M. | SC | 674 | 1.8 | 35 | 11.2 | 8 | 1.0 |
| 25. A. D. | SA | 660 | 0.4 | 27 | | 19 | 1.8 |
| 26. R. C. | SA | 671 | | 338 | | 1 | 0.0 |
| 27. M. M. | SS | 596 | 2.8 | 58 | 4.6 | 57 | 4.4 |
| 28. K. M. J. | SS | 687 | 1.8 | | 20.2 | 19 | 0.0 |
| Mean | | 649.0 | | | | 28.3 | 1.9 |
| S. D. | | ±34.9 | | | | ±24.4 | ±1.9 |

* The arterial $O_2$ saturation was 100 per cent in all these determinations.

TABLE V

*Results of cardiac catheterization*

| Patient No. | Hb. electrophoretic pattern | Wedge press. *mm. Hg* | PA press. *mm. Hg* | RV press. *mm. Hg* | RA press. *mm. Hg* | Pulm. vasc. res.* | Dye curve A.T.† | Cardiac index: Room air | Cardiac index: 100% $O_2$ | Total blood vol. *ml.* | Blood volume *ml./Kg.*‡ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2. J. A. | SS | 8.0 | 20/8 | 25/3 | 4 | 1.63 | 8 | 5.3 | 4.9 | 5,360 | 126 |
| 4. E. R. | SS | 15.0 | 43/12 | 49/9 | 8 | 2.04 | 9 | 5.4 | 4.3 | 6,503 | 121 |
| 6. W. Y. | SS | 8.0 | 21/10 | 19/3 | 8 | 1.02 | 8 | 4.8 | 3.9 | 4,280 | 73 |
| 8. R. C. R. | SS | 7.9 | 26/10 | 30/6 | 6 | 1.48 | 7 | 4.9 | 6.1 | 8,117 | 135 |
| 10. K. G. | SS | | | | 6 | | 8 | 3.6 | 2.6 | 3,585 | 72 |
| 12. E. D. | SS | 4.5 | 18/8 | 17/0 | 2 | 2.13 | 9 | 4.9 | 7.7 | | |
| 22. J. M. | SC | 8.0 | 20/10 | 23/6 | 7 | 1.37 | 10 | 3.5 | 4.4 | 4,251 | 84 |
| 24. H. M. | SC | 8.5 | 16/7 | 20/6 | 3 | 0.50 | 8 | 3.4 | 3.1 | 4,367 | 80 |
| 25. A. D. | SC | 3.0 | 14/6 | 17/0 | 3 | 2.72 | 10 | 3.1 | 3.8 | | |
| 26. R. C. | SA | 5.0 | 14/5 | 15/3 | 3 | 1.28 | 8 | 3.5 | 4.2 | | |
| Mean | | | | | | 1.53 | 8.5 | 4.24 | 4.5 | 5,209 | 98.7 |
| S. D. | | | | | | ±1.64 | ±0.9 | ±0.60 | ±1.4 | ±1,478 | ±23.4 |

* Pressure drop (pulmonary artery mean pressure-wedge pressure) in mm. of Hg per liters blood flow per minute per square meter.

† Appearance time (seconds) of T-1824 dye following injection into right atrium.

‡ Blood volumes were determined using both T-1824 dye and radioactive chromium for each value.

Anemia, of itself, has been generally accepted to be unaccompanied by arterial $O_2$ desaturation (6). However, a study by Ryan and Hickam (21) showed 10 severely anemic patients to have an average A-a gradient of 20.5 mm. Hg while breathing room air in comparison to an average gradient of 4.2 mm. Hg in six normal controls similarly studied. Similar results have been obtained on five anemic patients without sickling in this laboratory. It would appear, however, that the degree of desaturation in patients with sickle cell disease is relatively greater than that to be expected on the basis of anemia alone. In the present study, the average hemoglobin level in the sickle cell patients was 2.6 Gm. higher than that in the patients studied by Ryan and Hickam despite which the A-a gradients were much larger in the sickle cell group.

*Disturbances in diffusion*

The A-a gradients measured while low levels of oxygen were being breathed clearly indicate that defects in diffusion were not the major factor causing desaturation, although it is apparent that some loss of diffusing capacity had occurred in most of the patients. A pronounced abnormality of the diffusing capacity of the lungs, as evidenced by a gross increase in A-a gradient, was present in four patients (Nos. 1, 3, 11, 12). In all patients, the minute volume of ventilation remained close to normal, unaccompanied by subjective

TABLE VI

*Blood samples obtained simultaneously from multiple sites while breathing various oxygen mixtures*

| Patient | Left forearm vein: % Sat.* | Left forearm vein: $pO_2$† | Right forearm vein: % Sat. | Right forearm vein: $pO_2$ | Brachial artery: % Sat. | Brachial artery: $pO_2$ | Pulmonary artery: % Sat. | Pulmonary artery: $pO_2$ |
|---|---|---|---|---|---|---|---|---|
| No. 17 W. S. (Low $O_2$ breathing) | 74.6 | 41 | 21 | 16 | 66.4 | 35 | | |
| No. 8 R. C. R. (100% $O_2$ breathing) | 95.1 | 88 | 50 | 25 | 100 | 470 | 79 | 47 |
| No. 11 R. T. (100% $O_2$ breathing) | 76.8 | 45 | 100 | 256 | 100 | 596 | | |
| No. 10 K. G. (Room air breathing) | 88.2 | 62 | 58.1 | 32 | 90.6 | 64 | 61 | 33 |

* All oxygen saturations were determined by analysis for $O_2$ content and capacity on the Van Slyke manometric apparatus.

† All $O_2$ tension values were obtained by direct polarographic measurement.

disturbances, even at very low concentrations of inspired oxygen (9 per cent).

*Pulmonary veno-arterial shunting*

The most important mechanism producing desaturation appears to have been right-to-left shunting within the pulmonary parenchyma. This is indicated by the large A-a $pO_2$ gradients obtained while the patients breathed 100 per cent oxygen.

Two alternative causes for intrapulmonary shunting of blood from right-to-left in sickle cell patients appear possible. First, the shunting may be through anatomical veno-arterial channels which are present as a congenital malformation. Alternatively, the desaturation may be the result of blood traversing unaerated segments of the pulmonary parenchyma or moving through large vascular channels as a result of acquired changes in the lungs.

There is no concrete evidence for an increased incidence of generalized vascular abnormalities in sickle cell disease. Although dilated tortuous vessels have been demonstrated on microscopic pathological examination of the lungs as well as other organs in this disease (22), fistulous communications between arteries and veins are not described. Injection studies necessary to demonstrate such channels have, however, not been performed.

It is more likely that desaturation stems from pulmonary parenchymal alterations, presumably resulting from the frequent episodes of intrapulmonary difficulty to which these patients are subject. Pneumonia, pulmonary "sickle cell crises," and multiple pulmonary infarction have been invoked as a basis for the episodes.

The majority of the acute pulmonary attacks which have been observed in the subjects who form the background of this report have proved to be bacterial pneumonia with appropriate cultural confirmation and antibiotic response. There is also a possibility of the existence of some basic immunologic defect over and above the chronic anemic state.

The true nature of the clinical sickle cell crisis is obscure. It has been stated that, in order to compensate for the reduced oxygen carrying capacity found in any type of chronic anemia, an increased extraction of oxygen occurs peripherally and mixed venous blood has a lower than normal oxygen tension. This has been presumed to result in increased sickling within the central veins which, in turn, increases the viscosity of blood in the pulmonary vascular tree and slows the blood stream, resulting in sludging of cells with thrombosis in vessels and ischemic changes in surrounding tissues. The ischemia has been presumed to account for the symptoms which constitute a "crisis." Our studies do not support such a formulation. First, the values for pulmonary artery oxygen tension recorded while breathing room air (Table I) are higher, not lower, than would be expected from arterial $pO_2$ levels. Second, even when a marked decrease in the pulmonary arterial $pO_2$ with a concomitant increase in sickling was initiated by breathing of a low oxygen gas mixture, there was no evidence of any pathological process occurring within the lungs of any of these patients, either acutely or within a period of weeks from the time of study. Further, it is perhaps relevant that ventilatory studies provided no evidence of obliterative pulmonary vascular change; although all the patients showed decrease in vital capacity, the physiological dead space was normal in all.

*Peripheral arteriovenous shunting*

The presence of partially arterialized blood in certain peripheral veins is well established by these data. The demonstration of unusually high venous oxygen tensions in eight subjects while breathing 100 per cent $O_2$ (Table IV) is particularly striking. The phenomenon suggests a peripheral arteriovenous shunting mechanism and has been noted in normal subjects by Permutt and Isaacs (23). Whether the phenomenon is more marked or more frequent in patients with sickle cell disease than in normal subjects or patients with other types of anemia is unknown. Our own studies of normal subjects and of patients with anemia of other types (24) suggest that sickle cell disease may be associated with peripheral arteriovenous shunting of unusual magnitude and the various mechanisms that may be involved are currently under study.

*Intracardiac hemodynamic relationships*

The cardiac catheterization findings were similar to results previously reported by Leight,

Snider, Clifford, and Hellems (3). Despite the fact that cardiomegaly was present radiologically in almost all of these patients, the hemodynamic relationships, with one exception, were within normal limits. These findings do not substantiate the hypothesis that the cardiac enlargement in sickle cell disease is often due to cor pulmonale. Increased cardiac outputs and blood volumes, accompanied by normal pulmonary vascular resistances, indicate that the cardiac findings are principally those secondary to the high cardiac output. It is suggested that the mechanism producing these hemodynamic changes may be operative to a greater extent in sickle cell patients than in other comparably anemic patients because of a more profoundly decreased peripheral resistance, produced by extensive peripheral arteriovenous shunting.

## SUMMARY

Unsaturation of the arterial blood has been demonstrated in 16 out of 21 patients possessing S hemoglobin in various combinations.

By measuring A-a gradients on three levels of oxygenation, it has been determined that the desaturation is principally the result of intrapulmonary shunting of blood, although defects in diffusion also exist.

Increased cardiac outputs and blood volumes were accompanied by generally decreased pulmonary vascular resistances, suggesting that cor pulmonale is not common in these states.

The administration of very low concentrations of oxygen (9 to 11 per cent), while producing increased sickling in both the arterial and venous circulation, resulted in no discernible acute or chronic symptomatology.

## ACKNOWLEDGMENT

The aid of Dr. Elsie Smith and Dr. Leonor Oliveras during some of the studies, and the technical assistance of Mr. Bernard Williams and Mr. Claude McCain, Jr., are gratefully acknowledged.

## REFERENCES

1. Klinefelter, H. F. The heart in sickle cell anemia. Amer. J. med. Sci. 1942, **203**, 34.
2. Reinhard, E. H., Moore, C. V., Dubach, R., and Wade, L. J. Depressant effects of high concentrations of inspired oxygen on erythrocytogenesis. Observations on patients with sickle cell anemia with a description of the observed toxic manifestations of oxygen. J. clin. Invest. 1944, **23**, 682.
3. Leight, L., Snider, T. H., Clifford, G. O., and Hellems, H. K. Hemodynamic studies in sickle cell anemia. Circulation 1954, **10**, 653.
4. Halden, E. R., Sproule, B. J., Clarke, R. L., Muirhead, E. E., and Miller, W. F. Effects of $O_2$ breathing on the sickling phenomenon in vivo in sickle cell anemia and its variants (abstract). Amer. J. Med. 1957, **22**, 966.
5. Sproule, B. J., Halden, E. R., and Miller, W. F. Arterial oxygen desaturation in patients with S-type hemoglobin patterns (abstract). Clin. Res. Proc. 1957, **5**, 138.
6. Jensen, W. N., Rucknagel, D. L., and Taylor, W. J. Arterial oxygen unsaturation and possible mechanisms of its production in sickle cell anemia (abstract). J. clin. Invest. 1957, **36**, 905.
7. Lilienthal, J. L., Jr., Riley, R. L., Proemmel, D. D., and Franke, R. E. An experimental analysis in man of the oxygen pressure gradient from alveolar air to arterial blood during rest and exercise at sea level and at altitude. Amer. J. Physiol. 1946, **147**, 199.
8. Berggren, S. M. The oxygen deficit of arterial blood caused by nonventilating parts of the lung. Acta physiol. scand. 1942, **4**, Suppl. **11**.
9. Consolazio, C. F., Johnson, R. E., and Marek, E. Metabolic Methods. Clinical Procedures in the Study of Metabolic Functions. St. Louis, C. V. Mosby Co., 1951, p. 309.
10. Riley, R. L., Proemmel, D. D., and Franke, R. E. A direct method for determination of oxygen and carbon dioxide tensions in blood. J. biol. Chem. 1945, **161**, 621.
11. Sproule, B. J., Miller, W. F., Cushing, I. E., and Chapman, C. B. An improved polarographic method for measuring oxygen tension in whole blood. J. appl. Physiol. 1957, **11**, 365.
12. Scholander, P. F. Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. J. biol. Chem. 1947, **167**, 235.
13. Halden, E. R., Sproule, B. J., Clarke, R. L., and Muirhead, E. E. In preparation.
14. Crosby, W. H., and Furth, F. W. A modification of the benzidine method for measurement of hemoglobin in plasma or urine. Blood 1956, **11**, 380.
15. Comroe, J. H., Jr., Forster, R. E., DuBois, A. B., Briscoe, W. A., and Carlsen, E. The Lung. Chicago, Year Book Publishers, Inc., 1955, p. 81.
16. Riley, R. L., and Cournand, A. 'Ideal' alveolar air and the analysis of ventilation-perfusion relationships in the lungs. J. appl. Physiol. 1949, **1**, 825.
17. Singer, R. B., and Hastings, A. B. An improved clinical method for the estimation of disturbances

of acid-base balance of human blood. Medicine 1948, **27**, 223.

18. Wilson, R. H., Ebert, R. V., Borden, C. W., Pearson, R. T., Johnson, R. S., Falk, A., and Dempsey, M. E. The determinations of blood flow through nonventilated portions of the normal and diseased lung. Amer. Rev. Tuberc. 1953, **68**, 177.
19. Forssander, C. A. Alveolar air changes following inhalation of carbon dioxide during exercise and calculation of cardiac output. J. appl. Physiol. 1956, **8**, 509.
20. Miller, W. F., Johnson, R. L., Jr., and Wu, N. The half-second expiratory capacity test: A convenient means of evaluating the nature and extent of pulmonary ventilatory insufficiency. Dis. Chest 1956, **30**, 33.
21. Ryan, J. M., and Hickam, J. B. The alveolar-arterial oxygen pressure gradient in anemia. J. clin. Invest. 1952, **31**, 188.
22. Kimmelstiel, P. Vascular occlusion and ischemic infarction in sickle cell disease. Amer. J. med. Sci. 1948, **216**, 11.
23. Permutt, S., and Isaacs, M. Studies on oxygen saturation in superficial veins of the forearm (abstract). Clin. Res. Proc. 1957, **5**, 173.
24. Halden, E. R., and Sproule, B. J. Unpublished data.

# CLINICAL SCIENCE

*Edited for the Medical Research Society*
by W. MELVILLE ARNOTT

*Assistant Editors*
K. W. DONALD, HAROLD SCARBOROUGH, O. L. WADE

*Advisory Board*

E. A. CARMICHAEL
R. V. CHRISTIE
R. T. GRANT
J. MCMICHAEL
G. W. PICKERING
E. E. POCHIN
E. P. SHARPEY-SCHAFER
JANET VAUGHAN
W. C. WILSON
L. J. WITTS

**Volume 17, No. 2.** **Issued May, 1958**

**Clinical Signs Following Injury in Relation to Red Cell and Total Blood Volume.** By MARY R. FISHER.

**The Action of Fluorocortisone and Aldosterone on Sodium and Potassium Metabolism in Addison's Disease.** By D. S. MUNRO.

**Total Respiratory Compliance in Anaesthetized Subjects and Modifications Produced by Noxious Stimuli.** By P. R. BROMAGE.

**The Arterial Pressure "Run-off" as a Measure of Changes in Vascular Resistance in the Upper Limb.** By C. J. HAYTER and E. P. SHARPEY-SCHAFER.

**The Effect of Intraveous Calcium Gluconate on the Renal Excretion of Water and Electrolytes.** By P. FREEDMAN, R. MOULTON and A. G. SPENCER.

**Factors Determining the Dispersion of Dye in Indicator Dilution Curves in the Normal Mammalian Circulation.** By A. W. T. EDWARDS and P. I. KORNER.

**The Radioiodine Uptake of the Human Thyroid in Pregnancy.** By K. E. HALNAN.

**Factors Modifying Radiostrontium Excretion in Man.** By HERTA SPENCER, JOSEPH SAMACHSON, BERNARD KABAKOW and DANIEL LASZLO.

**Changes in the Blood-Clotting Defect in Christmas Disease After Plasma and Serum Transfusions.** By F. NOUR-ELDIN and JOHN F. WILKINSON.

**Metabolic Response of the Human Erythrocyte to Diabetic Acidosis.** By E. SHERWOOD JONES.

**Radio-active Isotope Measurements of Cardiac Output.** By RALPH SHACKMAN.

**Blood Flow in the Foot and Calf in the Elderly; a Comparison with that in Young Adults.** By M. J. ALLWOOD.

**Some Factors Affecting the Magnitude of the Pressor, Cardio-Accelerator and Pain Response to Cold of Heat-Acclimatized Subjects.** By G. C. WHITTOW.

**The Pulmonary Ventilation and Heart Rate During Exercise in Healthy Old Age.** By E. A. HARRIS and J. G. THOMSON.

**On the Inhibition of Plasma Fibrinolytic Activity by Exercised Ischaemic Muscles.** By H. C. KWAAN, R. LO and A. J. S. MCFADZEAN.

*Subscription Rate: £3.15.0. per volume.*

**Publishers: Shaw & Sons Ltd., 7, 8 & 9, Fetter Lane, Fleet Street, London, E. C. 4, England**